AN AUTOMATED SEMI-QUANTITATIVE BROAD DRUG SCREEN IN WHOLE BLOOD

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

University of Tasmania, June 1997

VOLUME I

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STATEMENT OF SOURCES

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Kathryn Campbell

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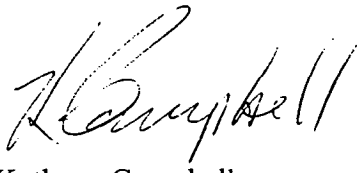

Kathryn Campbell

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ABSTRACT

Existing comprehensive drug screening procedures employ either batteries of analytical techniques which target specific drugs and structurally related drug groups, or relatively non-specific broad screens for either acidic or basic drugs which require additional confirmation of the identity of drugs detected. A single, automated, semi-quantitative gas chromatography/mass spectrometry (GC/MS) screening procedure for approximately 100 structurally and chemically diverse drugs in 2.5 mL of whole blood was developed and validated. A drug extraction strategy was developed which employed both liquid-liquid and solid-phase extraction techniques to produce four blood extracts for separate GC/MS analysis in selected ion monitoring (SIM) mode. The chromatograms produced were processed automatically by a novel macro which generated reports detailing the drugs identified and their approximate concentrations in blood.

Target drugs were selected for potential inclusion in the screening procedure on the basis of their ability to impair driving performance, their toxicological significance (forensic application) and their relative prevalence in the community (clinical application). As licit and illicit drug use in the community changes, these target drugs would change, however, those selected were representative of a range of chemical structures and functionalities broad enough to permit the development of a general analytical screening procedure which could be applicable to additional drugs.

The qualitative and quantitative characteristics of underivatised and derivatised (butyl- and pentafluoropropionyl-) target drugs were investigated to:-

1. determine retention indices and select suitable target *m/z* ions by which drugs would be identified;
2. establish quantitative parameters which were then used to estimate blood drug concentrations;
3. determine instrument detection limits and establish whether or not they were comparable with expected therapeutic drug blood concentrations; and
4. identify drugs not suitable for GC/MS analysis.

The recovery of 90% of target drugs was greater than 75% using the automated drug screening procedure.

The automated drug screening procedure was validated in blood specimens from a forensic laboratory and a hospital. The procedure identified 97% of drugs spiked at known concentrations in blood specimens prepared as part of a Proficiency Testing Program for Australian and New Zealand forensic laboratories. The developed screening procedure compared favourably with current toxicological methods routinely employed at the Government Analytical and Forensic Laboratory (Tasmania), identifying a greater number of drugs in the same blood specimens. During the analysis of blood specimens from hospital patients, it was found that the screening procedure was likely to identify the majority of drugs at therapeutic levels particularly if the blood taken was from an individual who had been previously administered the drug within two half-lives.

A pilot study which identified the drugs present in the blood of a limited sample of Tasmanian drivers involved in road traffic accidents indicated the applicability of the drug screen to studies which investigate the possible causal role of drugs in road accidents. The suitability of the screening procedure to other clinical and forensic applications was indicated.

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LIST OF ABBREVIATIONS

A/N	acidic and neutral drugs
amu	atomic mass unit
B/N	basic and neutral drugs
CI	chemical ionisation
DAD	diode array detector
DDD	defined daily dose/1000 head of population/day
DEA	diethylamine
DIP	direct insertion probe
DOA	drugs of abuse
ECD	electron capture detector
EI	electron impact
FID	flame ionisation detector
GAFL	Government Analytical and Forensic Laboratory
GC	gas chromatograph or chromatography
GC/MS	gas chromatograph/mass spectrometer or gas chromatography/mass spectrometry
HC	hydrocarbon
HPLC	high performance liquid chromatograph or chromatography
LLE	liquid-liquid extraction
M^+	molecular *m/z* ion
m/z	mass to charge ratio
MEOH	methanol
MSD	mass selective detector
MW	molecular weight
NPD	nitrogen-phosphorus detector
OC	on-column
PFP	pentafluoropropionyl
PFPA	pentafluoropropionic anhydride
RHH	Royal Hobart Hospital
RI	retention index or indices
RIA	radio-immuno assay
RTA	road traffic accident
SD	standard deviation from the mean
SIM	selected ion monitoring
SPE	solid-phase extraction
SPI	splitless programmable injector
TBAH	tetrabutylammonium hydroxide
temp. rate	rate of temperature increase (°C/min)
TIC	total ion current
TLC	thin layer chromatography
TMAH	tetramethylammonium hydroxide
UV	ultraviolet

LIST OF DRUG NAMES

LSD	lysergic acid diethylamide
MAM	mono-acetyl morphine
MDMA	methylenedioxymethylamphetamine
nordiazepam	desmethyldiazepam, metabolite of diazepam
PMA	*para*-methoxyamphetamine
THC	delta-9-tetrahydrocannabinol
THC-COOH	11-nor-carboxylic acid-delta-9-tetrahydrocannabinol

Preface

This thesis is contained in two volumes. Volume I contains the text and figures and Volume II contains the tables and reports. The separation of the text from the tables and reports was done to make it easier to read the thesis, for the following reasons:-

1. the thesis contains a large number of tables and reports;
2. often a single table or report consists of multiple pages;
3. tables are presented in both `portrait' and `landscape' orientations; and
4. some tables are referred to on more than one occasion throughout the text.

Having all tables and reports in Volume II allows the reader to have a table/report open for quick reference while reading the text, to orientate the second volume appropriately (landscape or portrait) and have a more manageable volume of text to read.

Volume II also contains Appendices A, B and C.

The pages of Volume II are not numbered sequentially as in Volume I but are numbered with the table/report identifier. For example, Table 7.2 consists of 7 pages and the third page of the table is numbered 7.2/3. In this way, each table can be quickly located and the reader can determine whether or not the table or report has multiple pages.

Chapter 1.

COMPREHENSIVE BROAD DRUG SCREEN IN WHOLE BLOOD

1.1 Introduction

The major aims of the research project were to:-

1. develop and validate an automated, semi-quantitative screening procedure for structurally and chemically diverse drugs in whole blood by benchtop GC/MS,
2. design a screening procedure particularly suited to forensic applications but which may also be useful in clinical settings, and
3. apply the screening procedure in a pilot study investigating drugs in Tasmanian drivers involved in road traffic accidents.

A comprehensive screen for a diverse range of drugs which both identifies drugs and indicates their concentration would be useful for:-

1. the treatment of patients in hospital Accident and Emergency wards,
2. the screening of employees and prison inmates with the view to detecting and preventing drug problems, and
3. the administration of law enforcement with regard to drug involvement in industrial and traffic accidents, breaches of State laws (eg. driving under influence of drugs, use of prohibited substances), and unexpected death (eg. overdose - accidental or otherwise).

In the design of any drug screening procedure, however, the particular needs and limitation of the laboratory in which it was to be employed, would need to be accommodated. Consequently, the needs and limitations of a Tasmanian forensic laboratory at the Government Analytical and Forensic Laboratory (GAFL) were considered for this study.

1.2 Needs and Limitations of a Forensic Laboratory

The forensic laboratory routinely analyses drugs in blood samples most often submitted by either the Police or the Coroner. The Police obtain fresh blood samples from individuals during their investigation of offences in connection with:-

1. driving under the influence of drugs,
2. use of prohibited substances,
3. motor vehicle accidents arising from possible driver impairment through drugs,
4. assaults, rapes and murders where drug use by either the victim and/or suspect impaired judgement and/or was otherwise related to the crime.

The Coroner obtains blood from individuals who have died unexpectedly, for example as a result of mechanical injury (eg. car accident, fall, suicide by hanging or gunshot), chemical injury (eg. drug poisoning, suicide by overdose, carbon monoxide poisoning) or homicide.

In such an environment the screening procedure is primarily a toxicological tool to

identify the drug/s present and their level in an individual's blood at a critical time. Although clients suspect that drugs are affecting individuals, very rarely do they have reliable evidence to identify the drug/s they suspect. Consequently, the ideal toxicological tool would detect any drug currently available, at a range of blood levels (sub-therapeutic to toxic).

In the majority of cases, the sample the client supplies is whole, haemolysed blood. Other than tissue samples, whole, haemolysed blood is the most difficult sample matrix from which to extract drugs as they must be separated from the protein, ruptured cell membranes and other interfering endogenous compounds which form the blood matrix. Although whole blood is a difficult specimen from which to extract drugs when compared with urine, serum or plasma, the client has difficulty obtaining these other specimens. For example, there is no current legislation which allows a urine specimen to be taken by the Tasmania Police without the consent of the individual being investigated. In coronial cases, often the bladder was voided on death and no urine could be obtained. The blood samples obtained from coronial cases range from being completely haemolysed to severely decomposed depending on how long the individual has been dead, and consequently, there is no chance that serum or plasma may be obtained from it. Blood taken under the Tasmania Road Safety (Alcohol and Drugs) Act is usually little better than post-mortem blood despite anticoagulants and preservatives being added. It is almost always haemolysed by the time the laboratory receives it, most often as a result of either poor mixing or poor storage prior to its arrival.

Drug levels determined in blood are of much greater interpretative value than drugs detected in a urine specimen. All that can be concluded with regard to drugs detected in a single 'snatched' urine specimen is that the individual was exposed to the drug in the recent past but no opinion could be given as to how much drug was taken, when it was last taken, or what effect that drug may have had on the individual. In contrast, pharmacokinetic drug data and information regarding the relationship between clinical effects of drugs and their concentration in blood plasma, allow a pharmacologist to give an educated opinion as to the likely effect detected drugs may have had on an individual at around the time their blood was taken. Consequently, for the forensic laboratory, a drug screen in whole blood is of the most practical and interpretative value.

The volume of blood submitted to the laboratory for analysis is sometimes restricted. The current legislation under which the Tasmania Police operate allows no more than 10 mL of blood to be drawn from the individual. This blood sample is drawn and separated by eye into three bottles, only one of which is available to the laboratory for analysis. Depending on the co-operativity and health of the individual and the availability of the hospital staff, significantly less than 3 mL of blood may be obtained for analysis. In most cases, the post-mortem blood volumes submitted by the Coroner are not limited unless there was either significant blood loss during or after death, or destruction of the body (eg. incineration, decomposition).

According to the scientific literature, broad drug screening and quantitation are generally not performed by the same assay on the same aliquot of blood unless the

assay specifically targets a group of structurally-related drugs. Consequently, multiple assays must be performed, using several aliquots of the blood, to identify and quantitate a diverse range of drugs. Such an approach to comprehensive drug screening requires that the laboratory:-

1. possess several major analytical instruments - eg. GC, HPLC and their appropriate detectors, GC-MS, and possibly RIA,
2. possess enough qualified staff to maintain and operate the instrumentation,
3. obtain sufficient specimen for analysis to both identify and quantitate drugs detected by the different techniques,
4. conduct analyses concurrently to ensure a timely result to the client.

Therefore, a screening procedure which both identifies and quantitates a large number of chemically diverse drugs in the same aliquot of blood would be most advantageous.

Quality control in any laboratory requires that the same analytical findings be obtained for a given specimen although a variety of different staff members perform the analyses and interpret the data. In the case of chromatograms generated by mass spectrometry, searching complex, multi-peak chromatograms for drugs is performed either:-

1. automatically by commercial or 'in-house' full-scan library search programs, or
2. manually by the experienced operator.

Commercially available library search programs (eg. those supplied with Hewlett Packard and Varian mass spectrometers) integrate and search all peaks in the chromatogram. The spectrum for each peak is compared with all spectra in the library and if a match is found, the 'goodness' of the match is reported as a percentage of an ideal match. Automating the search of a chromatogram for drugs ensures reproducibility and consistency of interpretation. However, although the search of each chromatogram is rapid and automated, there is generally no use made of the correlation between matched spectra and known drug retention time. In addition, unresolved peaks which give mixed mass spectra may lead to either false positives or negatives. Peaks which elute on the leading or tailing edge of a larger peak may not be integrated and may again result in false negatives.

In contrast, although manual searching of chromatograms against a library of drug mass spectra is much more laborious and time consuming, the expertise of the operator in interpreting the chromatogram provides special advantages. The experienced operator will detect peaks which appear on the shoulder of another peak. Similarly, the operator may have some knowledge of drug retention time and structure and may quickly dismiss a 'hit' if:-

1. the peak spectrum matched has the wrong retention time for that drug,
2. the suggested drug match could not possibly be chromatographed on the current system, or
3. the shape of the peak matched is different when compared with a known drug standard chromatographed on the same system.

Consequently, a screening procedure which automates the interpretation of complex multi-peak chromatograms and mimics the skills routinely employed by an

experienced operator would be highly advantageous.

The nature of forensic work often makes it the subject of legal scrutiny and confirmation of drug results by full-scan mass spectrometry is now almost a legal requirement. However, full-scan mass spectrometry is likely to lack the sensitivity of the initial screening technique. In the absence of full-scan mass spectra, confirmatory techniques which positively identify a drug by at least two different procedures satisfies the court. Again, this may be difficult if the sample volume is limited. Consequently, a drug screening technique which is rigorous enough to satisfy court requirements by confirming the presence of any drug it identifies would be highly desirable.

1.3 The Ideal Drug Screening Technique

The needs and limitations of the forensic laboratory outlined above would, in most cases, apply to any working laboratory (hospital, forensic, research). Therefore, the ideal comprehensive drug screening procedure has to satisfy several important criteria. The screen must:-

1. be broad enough to detect all drugs currently available and cover a sub-therapeutic to toxic range,
2. be suited to whole, haemolysed blood (< 3 mL),
3. both identify and quantitate the drugs detected,
4. be able to sustain court scrutiny,
5. not be reliant on the availability of multiple instruments and staff, and
6. provide automated interpretation of screening results to ensure timeliness and independence from the operator.

The ideal drug screening technique as described above has not yet appeared in the literature despite comprehensive research in the area for over 30 years. Although each criterion may be possible when approached independently of the others, in combination, they are impossible to satisfy. For example, no single analytical instrument is suited to the analysis of such a structurally diverse group of compounds as drugs. Drugs may have a polarity and structure which either prevent chromatography or ensure that chromatography will be poor. No single extraction technique provides great enough scope to extract all drugs due to limitations in the achievable pH range, solvent polarity etc. Some drugs will not be extracted while others will be extracted with poor or non-reproducible recoveries. The possible use of multiple extraction techniques and/or multiple instrumentations is restricted by the limited sample volume.

Consequently, in the development of an automated, quantitative, broad drug screening procedure, a compromise on the ideal must be made to satisfy achievable criteria. Realistic, achievable criteria can be described as follows.

1. A drug screen in whole, haemolysed blood.
2. The extraction of the majority of available drugs with at least one representative drug from each of the major drug classes (eg. diuretics, benzodiazepines, anti-depressants etc).
3. The detection of the majority of drugs at therapeutic blood concentrations.

4. Relatively high (>50%) and reproducible drug recovery.
5. Drug quantitation which provides an indication of whether the drug is present at sub-therapeutic, therapeutic or toxic levels.
6. The identification and confirmation of the majority of drugs by at least two different methods.
7. The chromatography of the majority of drugs with relatively good peak shape and with an instrument detection limit below the therapeutic range of the drug.
8. The automation of data analysis which provides greater specificity than commercially available library-based search programs and reproduces the skills of an experienced analyst.

1.4 Analytical Technique

1.4.1 Review of Current Literature

Most analytical techniques employed in drug detection can be broadly classified into two groups - chromatographic techniques and radio-immuno assay (RIA) techniques. The RIA techniques are most often seen in the hospital environment and the instrument and assays are generally commercially supplied. These methods are potentially expensive as their reagents have limited shelf-lives, and the screening assays can usually only be applied to urine samples, although there are some limited applications in serum. Radio-immuno assays require that separate analyses be performed for each individual drug or drug group rather than having one procedure which will encompass identification of drugs from different groups. These screening assays are not drug specific and consequently detection of a 'benzodiazepine' is reported rather than identifying a particular drug such as diazepam or nitrazepam. Similarly, quantitation of different drugs within the group is generally based on the calibration curve for a single representative drug.

There has been a vast amount of literature published in the area of drug analysis in biological materials over the past 30 years. Changing drug usage, the explosion in the number of new drugs (licit and illicit), increasing drug potency and greatly improved analytical instrumentation (more specific and sensitive detectors) over that period of time has made much of the early literature (pre-1985) obsolete. Even if the literature published in the last 15 years were extensively reviewed, the resulting document would be unmanageable. Consequently, the literature of this review will be considered in the light of the varied approaches used by researchers in drug analyses (extraction procedure, instrumentation) rather than documenting the specific details of the multitude of published analytical techniques. In addition, the review will be limited to chromatographic analyses in blood and blood products. The volume of literature devoted to the subject of drug analysis via chromatographic techniques indicates that it is constantly being explored, for example, an entire issue of the Journal of Chromatography was devoted to the chromatography of drugs and other toxic compounds.[1]

In large laboratories, batteries of analytical methods (RIA, TLC, GC, HPLC) which target groups of drugs are employed to conduct a comprehensive drug screen. With unlimited resources (time, staff, instrumentation, sample volume) an analyst could find in the current literature any number of procedures which identify and/or

quantitate either specific drugs, or groups of structurally-related drugs in biological fluids. In combination, these procedures could adequately provide a comprehensive and quantitative drug screen. Other than RIA techniques, there are a great variety of specific GC and HPLC based analyses for drug groups such as ß-blockers,[2-4] tricyclic anti-depressants,[5-9] benzodiazepines,[10-19] anti-inflammatories,[9,20,21] drugs of abuse,[22-26] diuretics,[27] oral anti-diabetics,[28] anticonvulsants,[29] and opiates.[30-32]

As an alternative to enlisting a number of drug assays in combination, there are many references to preliminary drug screening procedures employing GC. Earlier literature employed the flame ionisation detector (FID) particularly where drug identification was based on Kovats retention indices (RI).[33-35] There are many more studies which utilised a more specific detector - the nitrogen/phosphorus specific detector (NPD) with both dual and single column analyses.[5,36-50] GC-NPD provides a single analytical procedure which is sufficiently sensitive to detect large numbers of drugs. Nitrogen-containing drugs, endogenous blood products, blood and extraction contaminants, which elute from the capillary column as peaks, are described by specific retention times or RI. The retention time or RI is matched against those of drug reference standards run under the same chromatographic conditions. If the retention time of the eluted peak matches that of a drug standard, it is tentatively identified as that drug. However, the match is based on only a single criterion (retention time or RI) and, consequently, the drug screen is relatively non-specific compared with other techniques outlined below. The identity of the eluting peak requires additional confirmation. An endogenous blood product or other contaminant possessing characteristics which cause it to elute with the same retention time as a drug would be falsely identified as that drug. Similarly, should conditions arise which cause the retention time of an eluting drug to shift slightly (Section 8.4.1 and Appendix A.4) then it would not match that of the corresponding drug standard and a false negative would result.

Another important deficiency associated with GC-NPD based drug screening is that important non-nitrogenous drugs such as THC and its metabolites, and anti-inflammatory agents are not detected.[51] As with any chromatographic method, drugs which cannot be chromatographed under particular conditions go undetected unless their chromatography can be improved by derivatisation or altered capillary column polarity. One study also reported a large number of extraneous peaks which eluted close to the retention times of drugs,[52] making the interpretation of chromatograms difficult and further confirmatory techniques necessary. Many of the cited GC-NPD screening procedures did not give an indication of blood concentration as did the specific assays for individual drugs or drug groups.

The nature of forensic work often makes it the subject of legal scrutiny and confirmation of any drug identified is necessary. Recently, broad drug screening by diode array detectors (DAD) has become more prevalent in the literature.[19,53-63] Diode array detectors are useful confirmatory detectors as they provide a full ultraviolet (UV) spectrum of the compound eluting from the column. One study used automated detection based on both the UV spectrum and relative retention time of the eluting HPLC peak which were matched against a library of reference drug UV

spectra.[64] The drawback of DAD, however, is that many UV drug spectra are remarkably similar. This reduces the specificity of the screen and, consequently, for these drugs identification is again based on only one criterion (retention time). Another major disadvantage of DAD or UV detection is that it is too insensitive to detect many basic drugs as they possess such low specific extinction coefficients. Drug specific extinction coefficients cover an extremely wide range. For example, that of chlorpromazine in aqueous acid (λ_{max}=255 nm) is 1025, while that of methylamphetamine under the same conditions (λ_{max}=257 nm) is 12.[65] Consequently, the detectors have great sensitivity for some drugs while other drugs can be detected only at concentrations well above the therapeutic range. In the literature, HPLC-DAD is successfully employed in screening for acidic drugs which are generally administered in much larger doses than basic drugs.

The mass selective detector (MSD) is another useful confirmatory detector and well recognised and accepted in legal areas. The advantages and deficiencies of mass spectrometry are discussed below (Section 1.4.2). GC/MS has been used in screening procedures for specific drug groups such as opiates,[31] tricyclic anti-depressants,[6] drugs of abuse,[66,67] diuretics,[68] ß-blockers,[69,70] benzodiazepines,[71-74] anti-inflammatory analgesics,[75] neuroleptics,[76] and the subject is covered by reviews.[77-82] Some studies have automated data processing to identify drugs in multi-peak chromatograms on the basis of either unique mass spectra or groups of distinguishing *m/z* ions. In general however, automated drug searches involved matching the full-scan mass spectra of eluting drugs against commercial and purpose-built full scan mass spectral libraries (Section 8.1).

Just as there is a great variety of chromatographic instrumentation used for both broad drug screening and specific drug group analyses, so are there an even greater number of sample preparation and drug extraction techniques cited in the literature. The great majority of studies employ either liquid-liquid extraction (LLE) or solid-phase extraction (SPE) techniques where drugs are extracted from their matrix by partitioning them between two phases (Section 7.1). Despite the number of publications, many of the described procedures differ only subtly from others as researchers manipulate a limited number of parameters to optimise their procedure for the particular drug analyte. For example, LLE assays are described with a variety of buffers, pH conditions, extraction solvents, protein precipitation steps and extract clean-up steps.[36,38,39,50,53,60,83-90] Many pre-1980 papers described techniques for the extraction of drugs from tissues where drug concentrations are greater and, consequently, enzyme digestion techniques are also cited.[91] However, as instrument sensitivity improved with advancing technology, drug extraction from whole blood, serum and plasma eliminated the need for preliminary enzyme digestion. Solid-phase extraction techniques are described with a variety of packing materials, eluants, cartridge washing and pH adjusting solutions, and conditioning buffers.[4,5,13,18-20,41,45,88,92-102] The nature of SPE lends itself to automation.[9,103] Both types of extraction techniques may also have additional derivatisation steps prior to GC.[31,34,66,68,104,105]

It was not uncommon to find that important information with regard to the limitations and capabilities of the cited extraction techniques was not covered in all

publications. For example, data regarding which drugs could or could not be extracted by the technique, the detection limit and reproducibility of the assay, specific drug recovery, and the stability and reproducibility of derivatives formed, was often missing, incomplete, or provided for only a selection of drugs. Data was available for either a discrete number of drugs from a particular drug group or for representative drugs from specific drug groups while excluding some drugs from the same group which are more difficult to either extract or chromatograph. In short, not a single publication describing a comprehensive drug screen in whole blood was found that provided recovery and reproducibility data for a diverse group of drugs such as oxazepam, temazepam, morphine, paracetamol, salicylic acid and quinine or that indicated whether or not the technique did, in fact, extract all these drugs.

This general deficiency in the literature suggests a ninth criterion suitable for inclusion in the design of the achievable (and ideal) comprehenisive drug screen as follows.

The extraction technique details which drugs it can and cannot extract and indicates the extraction efficiency of the procedure for each drug.

1.4.2 The Mass-Selective Detector

The proposed drug screening technique is based on GC/MS analysis using a benchtop MSD. Mass spectrometry is concerned with the ionisation and subsequent fragmentation of compounds. The fragmentation is directed by the functional groups of the compound and occurs reproducibly. A result of reproducible fragmentation is a unique mass spectrum for each compound which displays the relative abundance of the ions produced by fragmentation and the determined mass to charge ratio (*m/z*). A widely used method of fragmentation is by electron impact (EI) in which the vaporised sample compounds are bombarded by a stream of high energy electrons.[65]

The detector can be operated in one of two ways as follows:-

1. it detects and records the sum of all the ions from the vaporised sample at any instant in time, displaying the total ion current (TIC), or
2. it detects only a selected number of specified target *m/z* ions in the vaporised sample while operating in selected ion monitoring (SIM) mode.

Total ion current mass spectra are invaluable for confirmation of drug identity as they display a fragmentation pattern that is unique for the drug and the molecular ion (M^+) may, sometimes, be stable enough to be apparent in the spectrum. The disadvantage of the TIC mode of operation is that sensitivity is reduced as a result of searching for up to 500 *m/z* ions, three times per second.

Many of the broad drug screening chromatographic procedures utilised NPD. This detector is more sensitive than an MSD operating in full-scan mode (TIC). However, when the MSD acquires data in the SIM mode, it has equivalent sensitivity and the more modern MSD instruments provide even greater sensitivity.

Although the MSD operating in SIM mode does not offer the same indisputable confirmation of drug identity provided by full-scan data, the detector will more reliably identify a drug than any other GC detector or the HPLC-DAD. Not only must the unknown compound elute from the column at the known retention time of

the drug standard but both the ratio and peak profiles of the target *m/z* ions monitored must mirror those of the drug reference standard. The target *m/z* ions selected for each drug generally incorporate both unique (eg. M^+) and abundant *m/z* ions giving specificity and sensitivity respectively. This additional evidence of the identity of a drug makes it very unlikely that false negatives will be reported.

In SIM mode, endogenous blood products and other sample contaminants go undetected as they elute from the column if they do not contain the target *m/z* ions currently monitored. As a consequence, chromatograms appear very 'clean' (smooth baseline) and the background is so low that even small peaks can be detected and integrated baseline to baseline.

The use of the MSD in the SIM mode, however, implies that only target drugs can be identified. The major disadvantage of SIM is that if a new, non-target drug was present in the blood sample, it would not be detected. The same could be said of other specific GC detectors (NPD or electron-capture detectors) which may not be sensitive to the new drug. The FID, however, would chromatograph the drug as a peak. In that instance, although the new drug may not match any reference drug standards currently run on that system, the operator would at least be aware of the presence of an extra peak which he/she would then have to determine by some means to be a drug and not simply an endogenous blood product or contaminant.

From a practical perspective, the MSD operated in SIM mode provides much greater specificity and information about an eluting compound than a non-specific FID while still matching the FID for the absolute number of compounds detected. The MSD will detect many more compounds than selective detectors such as NPD or ECD while still providing specific information with regard to *m/z* ions present. Benchtop MSDs are now common with up to 50 Hewlett Packard GC/MSDs now placed in laboratories around Australia and 11 GC/MS instruments in Tasmanian laboratories. Consequently, a comprehensive drug screen based on benchtop GC/MSD should provide an invaluable resource to Australian laboratories undertaking drug analyses.

1.5 Drug Selection

A comprehensive drug screening procedure employing benchtop GC/MSD operated in SIM mode requires that the screen target specific drugs. Consequently, a choice was made as to which drugs would be selected for investigation given that not all drugs currently available could be included in the time frame of the research project. It was important to the pilot study that all drugs of abuse (DOA) or potential abuse be identified by the screening procedure because of their capacity to alter mood and impair psychomotor functions and judgement. If the screening procedure was to be applicable in broader forensic and clinical settings, however, it must identify a wider range of drugs than psychotropic agents. Ideally, it should identify all drugs available to the community. Drug use in the community was indicated by the "1992 Australian Statistics on Medicines"[106] published in 1994. Figure 1.1 shows the introductory page of the publication which describes the source of the data, how the data is presented, and the units of measurement. Table 1.1 lists medications ranked in descending order by the defined daily dose/1000 head of population/day (DDD),

Introduction

The data contained in the 1992 ASM are drawn from two sources. The first is the Health Insurance Commission records of prescriptions submitted for payment of a subsidy under the Pharmaceutical Benefits and Repatriation Pharmaceutical Benefits Schemes. The second is an ongoing survey of a representative sample of computerised pharmacies, which provides an estimate of the non-subsidised use of prescription medicines in the Australian community. The usage of prescription medicines dispensed in public hospitals is not available in this report.

The units of measurement are the prescription and the defined daily dose per 1000 inhabitants per day (DDD/1000/day). The defined daily dose is established by the WHO Collaborating Centre for Drug Statistics Methodology on the basis of the assumed average dose per day of the drug, used for its main indication by adults. The drugs presented in this publication are arranged using the Anatomical Therapeutic Chemical classification system. For more detail on this classification and the unit of measurement, please read the chapter 'Information on the Australian Statistics on Medicines'.

The data are presented in two major tables. Table 1 includes estimates of 1992 community (ie, subsidised and non-subsidised) prescription numbers and cost (government and patient cost). Table 2 includes community prescription drug use, in DDDs/1000/day, for the years 1990 to 1992.

Figure 1.1: The introductory page of the "1992 Australian Statistics on Medicines" (ASM) compiled by the Commonwealth Department of Human Services and Health.

and indicates the major use of the medication by a brief description. An arbitrary cut-off point (DDD > 0.3) was selected to produce a more manageable number of drugs for consideration. Clearly, drug selection based on DDD alone would be inappropriate as many mineral supplements (calcium, iron), hormonal medications, anti-bacterial and anti-fungal agents, and topical or localised (non-systemic) medications appear with high DDD values but would have little significance to the Police or Coroner. In addition, it is immediately apparent that some of these medications (excluding some of the non-systemic medications) would not be suited to GC analysis. Absent from a list based on DDD alone would be:-

1. Some DOA (eg amphetamine and analogues, THC and metabolite).
2. Other drugs of significance in forensic applications such as drugs which are relatively toxic or lethal in overdose amounts (eg methadone, barbiturates, oxycodone, desipramine) and drugs with the potential for abuse (eg. pethidine, pseudoephedrine).
3. Common social drugs apart from DOA (eg caffeine, nicotine and its metabolite).
4. Some drug metabolites which may be present in an individual's blood for longer periods of time or at greater concentrations than the drug, or are pharmacologically active (eg. 7-amino flunitrazepam, THC-COOH, nordiazepam, nortriptyline).

Consequently, selection criteria in addition to DDD were required. Table 1.2 lists drugs which meet at least one of the following criteria.

1. Relatively prevalent in the population as indicated by DDD > 0.3.[106]
2. DOA potential.
3. Toxicologically significant - eg. drugs which commonly cause fatality, or with the potential to cause toxicity or fatality.[107,108]
4. Possessing pharmacological properties with the potential to affect driving performance or judgement - eg. centrally acting drugs (CNS), drugs affecting blood pressure or the cardiovascular system (CV), drugs affecting eyesight (O), drugs with undesirable side-effects which may impair vision, cause sedation or vertigo etc.[109-111]
5. Markers of an underlying disorder (eg. diabetes, epilepsy, Parkinsonism, gout).

The drugs listed in Table 1.2 provided the basis for drugs selected for further investigation. Initially, their chromatographic properties were explored (Chapters 3-5; Appendices A-B). Those drugs found to be successfully chromatographed at therapeutic blood levels (Chapter 6), were then investigated for their extraction potential via the extraction strategy which was developed (Chapter 7). Drugs which were found to both chromatograph and extract satisfactorily were included in the automated drug screening procedure which was then validated (Chapter 9) and applied to the 'drugs in drivers' pilot study (Chapter 10).

Although the drugs listed in Table 1.2 provided the basis for drug selection, clearly the list would alter over time with changing licit and illicit drug use in the community. The drugs listed, however, are representative of a range of chemical structures and functionalities and enable the development of a general analytical screening procedure which could be applicable to additional drugs.

References

1. "Chromatography of drugs and other toxic compounds."
Review Issue; J Chromatogr Biomed Appl (1990) 531, 1-500

2. "Rapid and simultaneous extraction of propranolol, its neutral and basic metabolites from plasma and assay by high performance liquid chromatography."
Harrison P, Tonkin A, Cahill C, McLean A; J Chromatogr (1985) 343, 349-358

3. "Improved screening method for β-blockers in urine using solid phase extraction and capillary gas chromatography mass spectrometry."
Leloux M; J Chromatogr (1989) 488, 357-367

4. "A strategy for the determination of β-blockers in plasma using solid phase extraction in combination of high performance liquid chromatography."
Musch G, Buelens Y, Massart D; J Pharmaceutical & Biomed Anal (1989) 7, 483-497

5. "A rapid and reliable method for extraction and assay of tricyclic antidepressant drugs in serum or plasma."
Roberts G, Hann C; Biomed Chromatogr (1986) 1, 49-53

6. "Improved tricyclic antidepressant assay by GC/MS."
Narasimhachari N, Friedal R, Saady J; Res Commun Psychology Psychiatry & Behav (1979) 4,477-494

7. "A Method for the determination of therapeutic and toxic concentrations of tricyclic antidepressant drugs in post-mortem fluids and tissues."
Hebb J, Crooks R, Caplan Y; J Anal Tox (1982) 6, 206-208

8. "Measurement of antidepressants using solid phase extraction and wide-bore capillary gas chromatography with nitrogen-selective detection."
Rifai N, Howlett C, Levtzow C, Phillips J; Therapeutic Drug Monit (1988) 10, 194-196

9. "Automated HPLC assays of fluoxetine and norfluoxetine in serum."
Nichols J, Charlson J, Lawson G; Clin Chem (1994) 40, 1312-1316

10. "An evaluation of HPLC for the screening and quantiation of benzodiazepines and acetominophen in postmortem blood."
Wong A, S. J Anal Tox. 7 (1983) 33-36

11 "Determination of 1,4- benzodiazepine and -diazepin-2-ones in blood by electron capture gas-liquid chromatography."
de Silva J; Anal Chem (1976) 48, 10-19

12. "Determination of benzodiazepines in full-blood after quantitative extraction with Extrelut® and high performance liquid chromatography with scanning ultra-violet detector."
Zweipfenning P, Kruseman K; Proceeding of 1990 TIAFT Meeting in Coppenhagen (1990) 327-336

13. "Rapid isolation of benzodiazepines with Sep-Pak® C_{18} cartridges."
Suzuki O, Seno H; J Forensic Sci (1988) 1249-1253

14. "An oxidative screening procedure for nanogram amounts of benzodiazepines and other drugs in blood."
Missen A; J Anal Tox (1977) 1, 224-226

15. "Analysis of benzodiazepines in chromatographic identification."
Chiarott M, Degiovanni N, Fiori A; J Chromatogr (1986) 358, 169-178

16. "High performance liquid chromatography determination of clonazepam in plasma using solid phase extraction."
Sallustio B, Kassapidis C, Morris R; Therapeutic Drug Monit (1994) 16,174-178

17. "Screening, identification and quantitation of benzodiazepines in postmortem samples by HPLC with photodiode array detection."
Lambert W, Meyer E, Xue-Ping Y; J Anal Tox (1995) 19, 35-40

18. "Solid-phase extraction of 1,4-benzodiazepines from biological fluids."
Casas-M, Berrueta-L-A, Gallo-B, Vicente-F; J Pharm Biomed Anal (1993) 11, 277-84

19. "A rapid solid-phase extraction and HPLC/DAD procedure for the simultaneous determination and quantification of different benzodiazepines in serum, blood and post-mortem blood."
Musshoff F, Daldrup T; Int J Legal Med (1992) 105, 105-9

20. "Rapid extraction of anti-inflammatory drugs in whole blood for high performance liquid chromatography analysis."
Moore C, Tebbett I; Forensic Sci Int (1987) 34, 155-158

21. "High performance liquid chromatographic assay for ibuprofen in whole blood using solid phase extraction."
Sochor j, Klimeš J, Zahradnicek M; J Chromatogr B (1994) 654, 282-286

22. "Identification of major drugs of abuse using chromatography."
Gough T, Baker P; J Chromatogr Sci (1982) 20, 289-329

23. "Confirmatory test for drugs in the workplace by gas chromatography-mass spectrometry."
Goldberger B, Cone E; J Chromatogr. A 674 (1994) 73-86.

24. "Solid phase extraction and HPLC-UV confirmation of drugs of abuse in urine."
Ferrara S, Tedeschi L, Frison G; J Anal Tox (1992) 16, 217-222

25. "Identification of major drugs of abuse using chromatography: an update."
Gough T, Baker P; J Chromatograph Sci (1983) 21,145-153

26. "Gas-liquid chromatography of phencyclidine in serum, with nitrogen-phosphorus detection."
Werner M, Hertzman M, Pauley C; Clin Chem (1986) 32, 1921-4

27. "Estimation of diuretic drugs in biological fluids by HPLC."
Herráez-Hernández R, Campins-Falcó P, Sevillano-Cabeza A; Chromatographia (1992) 33, 177-185

28. "Determination of anti-diabetic agents in human body fluids using high performance liquid chromatography."
Hill H, Chamberlain J; J Chromatogr (1978) 149, 349-358

29. "Simultaneous very fast liquid chromatographic analysis of ethosuximide, primidone, phenobarbital, phenytoin and carbamazepine in serum."
Kabra A, Nelson M, Marton L; Clin Chem (1983) 29, 473-476

30. "Morphine concentration in blood and organs in cases of fatal poisoning."
Felby S, Christensen H, Lund A; Forensic Sci. 3(1974) 77-81

31. "Gas chromatography-mass spectrometric determination of morphine, codeine and 6-mono-acetyl-morphine in blood extracted by solid phase."
Schuberth J, Schuberth J; J Chromatogr Biomed Appl (1989) 490, 444-449

32. "Simultaneous solid-phase extraction and chromatographic analysis of morphine and hydro-morphone in plasma by high performance liquid chromatography with electrochemical detection."
Bouquillon A, Freeman D, Moulin D; J Chromatogr (1990) 577, 354-357

33. "Applications of capillary gas chromatography in routine toxicological analyses."
Anderson W, Staffor D; J High Resolution Chromatogr and Chromatogr Commun. (1983) 6,246-254

34. "A rapid screening procedure for acidic and neutral drugs in blood by high resolution gas chromatography."
Sharp M; J Anal Tox (1987) 11, 8-228

35. "Comparison of drug retention indices determined on packed, wide-bore capillary and narrow-bore capillary columns."
Japp M, Gill R, Osselton M; J Forensic Sci (1987) 32,1574-1586

36. "Automated quantitative screening for acidic and neutral drugs in whole blood by dual column capillary gas chromatography."
Ojanperä I, Rasanen I, Vuori E; J Anal Tox (1991) 15, 204-208

37. "A new rapid gas chromatographic method for the detection of basic drugs in post-mortem blood."
Pierce W, Lamoreaux T, Urry F; J Anal Tox (1978) 2, 26-31

38. "Screening for neutral and basic drugs in blood by dual fused-silica column chromatography with nitrogen-phosphorus detection."
Turcant A, Premel-Cabic A, Cailleux A, Allain P; Clin. Chem. 34 (1988) 1492-1497

39. "A single-step extraction for screening whole blood for basic drugs by capillary GC/NPD."
Cox R; J Anal. Tox. 13 (1989) 224-228

40. "Use of dual column fused-silica gas chromatography in combination with detector response factors for analytical toxicology."
Perrigo B, Peel H, Ballantyne D. J Chromatogr. (1985) 341, 81-88

41. "A rapid isolation of acidic and basic drugs."
Cordonnier J, Van Den Heede M, Heyndrickx A; Int Anal (1987) 3, 28-34

42. "Determination of basic drugs extracted from biological matrices by means of solid-phase extraction and wide bore capillary gas chromatography with nitrogen phosphorus detector."
Chen X, Franke J, Wijsbeck J, deZeeuw R; J Anal Tox (1994) 18, 150-153

43. "Capillary gas chromatographic drug screen for use in forensic toxicology."
Drummer O, Horomidis S, Koortis S; J Anal Tox (1994) 18, 134-138

44. "Simultaneous screening and quantitation of alpidem, zolpidem, bispirone and benzodiazepines by dual-channel gas chromatography using electron capture and nitrogen-phosphorus detection after solid phase extraction."
Gaillard Y, Gay-Montchamp J, Ollagnier M; J Chromatogr Biomed Appl (1993) 197-208

45. "Toxicological analysis of whole blood samples by means of bond-elut certify columns and gas chromatography with nitrogen-phosphorus detection."
Zweipfenning P, Wilderink A, Horsthuis P, Franke J, de-Zeeuw R; J Chromatogr A (1994) 674, 87-95.

46. "Determination of basic drugs extracted from biological matrices by means of solid-phase extraction and wide-bore capillary gas chromatography with nitrogen-phosphorus detection."
Chen X, Franke J, Wijsbeek J, de-Zeeuw R; J Anal Toxicol (1994) 18 150-3,

47. "Screening of basic drugs in biological samples using dual column capillary chromatography and nitrogen-phosphorus detectors."
Watts V, Simonick T; J Anal Tox (1986) 10,198-204

48. "Screening of basic, neutral and weakly acidic drugs from post mortem blood using solid phase extraction procedure and GC."
A, Wikström M; TIAFT - Proceeding of the 30th International Meeting (1992)232-235

49. "Evaluation of a screening procedure for basic and neutral drugs: n-butyl chloride extraction and megabore capillary gas chromatography."
Sharp M: Can Soc Forensic Sci J (1986) 19, 83-101

50. "Testing for basic drugs in biological fluids by solvent extraction and dual capillary GC/NPD."
Fretthold D, Jones P, Sebrosky G, Sunshine I: J Anal Tox (1986) 10,10-14

51. "Automated screening procedure using gas chromatography mass spectrometry for identification of drugs after their extraction from biological samples."
Neill G, Davies N, McLean S; J Chromatogr Biomed Appl (1991) 565, 207-224

52. "Drugs, alcohol and road accidents in tasmania."
McLean S, Parsons R, Chesterman R; Med. J Aust. (1987) 147, 6 - 1

53. "Rapid screening for 100 drugs and metabolites using cation exchange solid phase extraction and high performance liquid chromatography with diode array detection."
Logan B, Stafford D; J Anal Tox (1990) 14, 154-158

54. "HPLC photodiode array UV detection for toxicological drug analysis."
Hill D, Langever K; J Liquid Chromatogr (1987) 10, 377-409

55. "Evaluation of a photodiode array/high performance liquid chromatographic-based system for the detection and quantitation of basic drugs in postmortem blood."
Koves E, Wells J; J Forensic Sci JFSCA (1992) 37, 42-60

56. "Identification power of a standardised HPLC-DAD system for systematic toxicological analysis."
Maier R, Bogusz M; J Anal Tox (1995) 19, 79-83

57. "Systematic toxicological analysis of basic drugs by gradient elution of an alumina-based HPLC packing material under alkaline conditions."
Lambert W, Meyer E, De Leenheer A; J Anal Tox (1995) 19, 73-78

58. "Extraction of basic drugs from whole blood and determination by high performance liquid chromatography."
Bernal J, Delnozal M, Rosas V, Villarino A; Chromatographia (1994) 38, 9-10

59. "A class independent drug screen in forensic toxicology using a photodiode array detector."
Drummer O, Kotsos A, McIntyre I; J Anal Tox (1993) 17, 225-226

60. "Use of high-performance liquid chromatography diode array detection in forensic toxicology."
Koves E; J Chromatogr (1995) 692,103-119

61. "Liquid chromatography with photodiode array spectophotometric detection in the forensic sciences."
Logan B; Analytica Chimica Acta (1994) 288,111-122

62. "A new drug screening system multi-hplc by high performance liquid chromatography using multiwavelength uv detector and its clinical application."
Suzaki S, Yamamoto Y, Hayashida M; Toxicology Methods (1994) 4, 122-134

63. "Systematic toxicological analysis using HPLC/DAD."
Tracqui A, Kintz P, Mangin P; J Forensic Sci (1995) 40, 254-262

64. "Forensic application of an automated drug-profiling system."
Kalasinsky K, Schaefer T, Binder S; J Anal Tox (1995) 19, 412-417

65. "Clarke's Isolation and Identification of Drugs."
Moffat A.C (Ed.) Second Edition (1986) The Pharmaceutical Press, London.

66. "GC/MS assays for abused drugs in body fluids."
Foltz R, Fentiman A; NIDA Research Monographs (1980) 32

67. "Screening and confirmation of drugs of abuse."
Analytical Systems, Division of Marion Laboratories Inc. (1987) 51-79

68. "Detection of diuretics in horse urine by GC/MS."
Hagedorn H, Schulz R; J Anal Tox (1992) 16, 194-198

69. "Simultaneous determination of propranolol or metoprolol in the presence of butyrophenones in human plasma by gas chromatography with mass spectrometry."
Quaglio M, Bellini A, Minozzi L; J Pharmaceutical Sci (1993) 82, 87-90

70. "Identification and differentiation of β-blockers and their metabolites in urine by computerised gas chromatography-mass spectrometry."
Maurer H, Pfleger K; J Chromatogr (1986) 382, 147-165

71. "Quantitative liquid chromatography, thermal/tandem mass spectrometry (LC/TSP/MS/MS) analysis of some thermolabile benzodiazepines in whole blood."
Verweij A, Lipman P, Zweipfenning P; Forensic Sci Int (1992) 54, 67-74

72. "Determination of 1,4- and 1,5- benzodiazepines in urine using computerised gas-chromatography-mass-spectrometric technique."
Maurer H, Pfleger K; J Chromatogr (1981) 222, 409-419

73. "Identification and differentiation of benzodiazepines and their metabolites in urine by computerised gas chromatography mass spectrometry."
Maurer H, Pfleger K; J Chromatogr Biomed Appl (1987) 422, 86-101

74. "Benzodiazepines identified by capillary gas chromatography-mass spectrometry, with specific ion screening used to detect benzophenone derivatives.
Jones C, Wians F, Martinez L, Merritt G; Clin. Chem. (1989) 35, 1394-1398

75. "Screening procedure for detecting anti-inflammatory analgesics and their metabolites in urine using a computerised gas-chromatography-mass- spectrometric technique."
Maurer H, Pfleger K; Fresenius Z'Anal Chem (1984) 317, 42-52

76. "Screening procedure for detecting butyrophenone and bisfluorophenyl neuroleptics in urine using a computerised gas chromatography-mass spectrometric technique."
Maurer H, Pfleger K; J Chromatogr Biomed Appl (1983) 272;75-85

77. "Chromatographic techniques - the basis of doping control."
Mueller R, Grosse R, Thieme L; J Chromatogr B (1995) 674, 1-11

78. "Quantitative selected ion monitoring (QSIM) of drugs and/or drug metabolites in biological matrices."
Garland W, Powell M; J Chromatogr Sci (1981) 19,392-434

79. "Systematic toxicological analysis of drugs and their metabolites by gas chromatography-mass spectrometry."
Maurer H; J Chromatograph Biomed Appl (1992) 580, 3-41

80. "Scope and limitation of a general unknown screening by gas chromatography-mass spectrometry in acute poisoning."
Köppel C, Tenczer J; J Am Soc Mass Spectrom (1995) 6, 995-1003

81. "A comprehensive GC/MS drug screening procedure."
Ullucci P, Cadoret R, Stasiowski P; J Anal Tox (1978) 2,33-38

82. "Automated identification by computer of the mass spectra of drugs in urine or serum extracts."
Smith N; J Anal Tox (1994) 18,16-21

83. "Screening biological samples for underivatized drugs using a splitless injection technique on fused silica capillary column gas chromatograph."
Ehresman D, Price S; J Anal Tox (1985) 9, 55-62

84. "Toxicological determination of benzodiazepines in biological fluids and tissues by flame ionisation gas chromatography."
Baselt R, Stewart C; J Anal Tox (1977) 1, 10-13

85. "Extraction of basic drugs from whole blood and determination by high performance liquid chromatography."
Bernal J, Nozal M, Rosas V, Villarino A; Chromatographia (1994) 38, 617-623

86. "Sample preparation for organic acids in biological fluids."
Leibich H; Analytica Chimica Acta (1990) 236, 121-130

87. "Whole blood deproteinisation for drugs screening using automatic pipettors."
Collins C, Muto J, Spiehler V; J Anal Tox (1992) 16, 340-342

88. "Review - Sample preparation for biomedical analysis."
McDowall R; J Chromatogr (1989) 492, 3-58

89. "Application of a simple extraction procedure using aqueous ammonia to the analysis of basic drugs in blood by gas chromatography."
Sims D, Felgate P, Felgate H, Lokan R; Forensic Sci Int (1991) 49,333-42

90. "A rapid method for the identification of acidic, neutral and basic drugs in postmortem liver specimens by Toxi-Lab®."
Whitter P, Cary P; J Anal Tox (1986) 10,68-71

91. "Comparative evaluation of some enzyme digestion procedures in the release of basic drugs from tissues."
Shankar V, Damodaran C; J Anal Tox (1987) 11, 164-167

92. "A rapid extraction method for acidic drugs in haemolysed blood."
Ford B, Vine J, Watson T; J Anal Tox (1983) 7, 116-118

93. "A simplified procedure for the isolation, characterisation and identification of weak acid and neutral from whole blood."
Anderson W, Fuller D; J Anal Tox (1987) 11, 198-207

94. "Isolation of basic drugs from plasma using solid phase extraction with cyano-propyl bonded phase."
Musch G, Massart D; J Chromatogr (1988) 529, 209-222

95. "Solid-phase extraction for the screening of acidic, neutral and basic drugs in plasma using a single-column procedure on Bond Elut Certify."
Chen X, Wijsbeek J; J Chromatogr (1990) 529, 161-166

96. "Knowledge-based system for the automated solid phase extraction of basic drugs from plasma coupled with their liquid chromatographic determination."
Hubert P, Chiap P, Moors M; J Chromatogr A (1994) 605, 87-99

97. "Solid phase extraction of drugs from biological tissues - A review."
Scheurer J, Moore C; J Anal Tox (1992) 16, 264-269

98. "A simplified thin layer chromatographic system for the detection of commonly abused basic drugs."
Blakesley J, Wood D, Howse C; Ann Clin Biochem (1987) 24, 508-510

99. "Screening and confirmation of drugs in horse urine by a simple column extraction procedure."
Singh A, Ashraf M, Granely K; J Chromatogr (1989) 473, 215-226

100. "Trends in techniques for the extraction of drugs and pesticides from biological specimens prior to chromatographic separation and detection.
Furton K, Rein J; Analytica Chimica Acta (1990) 236,99-114]

101. "A single column procedure on Bond Elut Certify for systematic toxicological analysis of drugs in plasma and urine."
Chen X, Wijsbeek J, Franke J, de-Zeeuw R; J-Forensic-Sci (1992) 37, 61-71

102. "Analysis of drugs from biological fluids using disposable solid phase columns."
Doyle E, Pearce J, Picot V, Lee R; J Chromatogr (1987) 411,325-33

103. "Pitfalls and solutions in the development of a fully automated solid-phase extraction method for drug screening purposes in plasma and whole blood."
Chen X, Franke J, Ensing K, Wisjbeek, de Zeeuw R; J Anal Tox (1993) 17, 421-426

104. "Pentafluorobenzoyl derivatives of doping agents."
Dekbeke C, Debackere M; J Chromatogr Biomed Appl (1983) 273, 141-149

105. "Screening of beta-blockers in human serum by ion-pair chromatography and their identification as methyl or acetyl derivatives by gas chromatography-mass spectrometry."
Siren H, Saarinen M, Hainari S, Lukkari P, Riekkola M; J Chromatogr (1993) 632, 215-27

106. "1992 Australian statistic on medicines."
Commonwealth Department of Human Services and Health (1994) Australian Government Publishing Service, Canberra; 157-242

107. "Survey of admissions due to drug overdose at the Royal Hobart Hospital."
Drummond S; University of Tasmania; Tasmanian School of Pharmacy MSP300 Project (1994) 1-27

108. "Suicide profiles in Tasmania 1995."
Ryall J; Department of Community and Health Services Tasmania; Report to Program Manager of Mental Health Coordination Unit. (1995) 1-12

109. "1996 MIMS Annual"
Carroll, P (Chairman of Editorial Board) 20th Edition -Australian (1996) Tien Wah Press (Pte.) Ltd. Singapore

110. "Australian Prescription Products Guide."
Thomas J (Ed) 23rd Edition (1995) Australian Pharmaceutical Publishing Company Limited; Australia.

111. "Martindale. The Extra Pharmacopoeia"
Reynold J (Ed.) 30th Edition (1993) The Pharmaceutical Press, London

Chapter 2.

GENERAL PROCEDURES

2.1 Overview

The screening procedure targets selected drugs. It consists of two separate extraction procedures (LLE and SPE). Drugs isolated from whole blood by the extraction procedure are analysed by GC/MS in SIM mode. For each blood specimen, four separate GC extracts (underivatized and derivatised extracts from LLE and SPE) are analysed. These analyses, in combination, identify target drugs and, in many cases, confirm their presence by an alternate method.

Chromatograms are processed by a macro which automatically searches for target drugs and indicates 'hits' detected. The estimated blood concentration of the suspected 'hit' is automatically calculated on the basis of predetermined quantitative parameters. The results from each of four reports generated by the macro are collated onto a summary sheet (Section 8.3.3) and the drugs present in the blood sample are decided by the analyst. The analyst requires only a little experience to interpret the macro reports correctly.

The procedure is suitable for the analysis of most common acidic, neutral and basic drugs including drugs of abuse in whole blood. Additional drugs can be added to the screening procedure as new target drugs arise. The procedure is sensitive enough to detect most drugs at sub-therapeutic and therapeutic levels although some drugs will be detected only at greater than therapeutic levels.

2.2 Reagents

2.2.1 Liquid-liquid Extraction (LLE)

Saturated ammonium chloride
Ethyl acetate (HPLC grade)
Acetonitrile (HPLC grade)
Hexane saturated with acetonitrile (HPLC grade)
Chloroform GC injection solvent; (Section 2.2.5.7)
HC marker solution; (Section 2.2.5.5)

2.2.2 Solid-phase Extraction (SPE)

Phosphate buffer (0.1 M); (Section 2.2.5.1)
Bond Elut 'Certify' extraction column (130 mg of mixed mode sorbent)
Acetic acid (0.01M); (Section 2.2.5.2)
Deionised water
Methanol (HPLC grade)
Acetone:chloroform (1:1; HPLC grade)
Freshly prepared 5% ammonia in dichloromethane:isopropanol (80:20; HPLC grade)
Chloroform GC injection solvent; (Section 2.2.5.7)
HC marker solution; (Section 2.2.5.5)

2.2.3 Butylation

Dimethylformamide
Trimethylammonium hydroxide (TMAH - 0.1 M) in methanol; (Section 2.2.5.4)
n-butyl iodide
Chloroform GC injection solvent; (Section 2.2.5.7)

2.2.4 Pentafluoropropionylation

Dried acetonitrile; (Section 2.2.5.12)
Pentafluoropropionic anhydride (PFPA)
Dried ethyl acetate GC injection solvent; (Section 2.2.5.8)

2.2.5 Reagent Preparation

2.2.5.1 *0.1 M Phosphate buffer*

KH_2PO_4 (6.805 g) was dissolved in 500 mL deionised water and adjusted to pH 6.0 ± 0.05 with a KOH solution (3-4 chips of KOH in ≈ 10 mL deionised water). The solution was stored at 4°C and discarded after 1 month.

2.2.5.2 *0.01M Acetic acid*

A 1.0 mL aliquot of glacial acetic acid (1.0 M) was diluted to 100 mL with deionised water. The solution was stored at room temperature and discarded after 12 months.

2.2.5.3 1.0 *M Acetic acid*

Glacial acetic acid (5.75 mL) was diluted to 100 mL with deionised water. The solution was stored at room temperature and discarded after 12 months.

2.2.5.4 *0.1M TMAH in methanol*

TMAH (18.12 g) was dissolved in 100 mL MEOH and stored indefinitely at room temperature.

2.2.5.5 *HC Marker solution*

A 10 mL aliquot of stock HC marker solution was diluted with 10 mL hexane and stored indefinitely at 4°C in a silanised glass culture tube.

2.2.5.5 *Stock HC Marker solution*

Consecutive, even-numbered *n*-alkanes (C_{10} to C_{36} - 5 mg) was dissolved in 100 mL of hexane and stored indefinitely at 4°C.

2.2.5.7 *Chloroform GC injection solvent*

A 50 mL aliquot of Injection Solvent HC solution (Section 2.2.5.9) was diluted with 50 mL of chloroform. The solution was stored at room temperature and discarded after 12 months.

2.2.5.8 *Dried Ethyl acetate GC injection solvent*:

A 50 mL aliquot of Injection Solvent HC solution (Section 2.2.5.9) was diluted with 50 mL of ethyl acetate and dried over molecular sieve (Section 2.2.5.11). The solution was stored at room temperature and discarded after 12 months.

2.2.5.9 *Injection Solvent HC solution*
C_{10}, C_{12} and C_{14} (5 mg) were dissolved in 100 mL of hexane. The solution was not stored but immediately and completely used in the preparation of chloroform and ethyl acetate injection solvents (Sections 2.2.5.7 and 2.2.5.8).

2.2.5.10 *Performance Test Mix*
Drug solutions (approximately 1 mg/mL) were added as indicated to 4 mL of Stock HC Maker solution (Section 2.2.5.5) and diluted to 10 mL with hexane. Fenfluramine (0.1 mL), cotinine (0.2 mL), phenobarbitone (0.15 mL), methadone (0.1 mL), moclobemide (0.1 mL), promethazine (0.1 mL), codeine (0.15 mL), nordiazepam (0.15 mL), disopyramide (0.15 mL), temazepam (0.5 mL), metoclopramide (0.2 mL), felodipine (0.1 mL), quinine (0.4 mL), prednisolone (0.4 mL), diltiazem (0.1 mL), prochlorperazine (0.2 mL), thioridazine (0.15 mL), verapamil (0.1 mL). The solution was stored indefinitely in a silanised glass tube at -18°C.

2.2.5.11 *Silanising Reagent*
Dimethyldichlorosilane (Aldrich) was prepared as a 5% solution in toluene. The reagent was stored in glass, and used repeatedly. The reagent was discarded if any yellow discolouration occurred.

2.2.5.12 *Dried Solvents*
Solvents were dried over molecular sieve (BDH No. 4A, 1/16″; approximately 15g/200 mL solvent) which had been first washed with the appropriate solvent and oven dried (60 min at 100°C).

2.2.5.13 *Reference Drug Solutions*
Drugs were of pharmaceutical grade or better and supplied in either free drug or salt forms. Stock solutions (approximately 1 mg/mL) of free drug were prepared in either chloroform, methanol, dimethylformamide (DMF) or chloroform:DMF (2:1) depending on the solubility of individual drugs.

Stock solutions of drug salts were prepared as follows.
Approximately 10 mg equivalent of free drug was dissolved (5 mL) in either aqueous acid (adjusted with 0.5 N HCl; pH < 3) or aqueous alkali (adjusted with a diluted ammonia solution; pH > 9); free drug was extracted into 2 x 4 mL aliquots of chloroform; the solution was made up to a 10 mL volume which became the stock solution; the aqueous phase was analysed by UV (scanning from 360 to 200 nm) for remaining drug and re-extracted if drug was detected; the concentration of free drug in stock solutions was approximately 1 mg/mL.

2.2.5.14 *Working Drug Solutions*
Groups of up to ten drugs obtained from their reference drug solutions were combined in a single solution to produce working drug solutions in chloroform at concentrations of approximately 20, 60, 100, 200, 300, 400, and 800 pg/μL, and 2, 4, 20 and 50 ng/μL.

2.3 Apparatus

2.3.1 Drug Extraction

silanised 15 mL glass culture tubes + teflon lined screw-caps (Kimble); (Section 2.3.3)
disposable plastic pipettes (Samco)
rotary mixer; radius of 20 cm; 40 rpm is equivalent to 0.35 g
12 port vacuum manifold (Alltech)
sonic bath
centrifuge; radius of 20 cm; 2000 rpm is equivalent to 895 g
heating block and nitrogen manifold
50 µL transfer pipette and disposable tips

2.3.2 Drug Derivatisation and GC/MS Injection

2 mL screw-capped reaction vials with teflon liners (Alltech)
2 mL autosampler vials (Alltech)
200 µL autosampler vial inserts (Alltech)

2.3.3 Preparation of Silanised Glassware

Glassware (culture tubes and caps) were washed with general detergent, rinsed with deionised water and oven dried. Approximately 3 mL silanising reagent (Section 2.2.5.11) was added to each tube, which was then capped and placed on a rotary mixer for 3-4 h. The silanising reagent was retained for future use. The silanised tubes and caps were rinsed with two washes of methanol and oven dried.

2.4 GC/MS Analyses

2.4.1 Instrumentation

Low resolution electron impact mass spectrometric analyses used a Hewlett Packard (HP) 5890 gas chromatograph (GC), with split/splitless injector, coupled with a HP5970 mass selective detector (MSD) with open split interface from column to MSD, and operated in either full scan or selected ion mode by PC based operating software. Sample injection was automated with a HP 7673A autosampler.

2.4.2 Gas Chromatography

All analyses unless otherwise specified used a 25 m cross linked methyl silicone (HP-1) high performance capillary column with 0.32 mm i.d. and 0.17 µm film thickness. A 50 cm length of precolumn identical to the separating column, was attached to the separating column by a column connector (capillary mini-union; 0.5 mm i.d; GLT™, SGE Analytical Products). The injector port contained a borosilicate glass liner (0.4 mm i.d., 990 µL) packed with a silanised glass wool plug unless otherwise specified. Prior to use, the liner was washed with detergent and rinsed, baked in a muffle furnace at 520°C for several hours and then silanised with silanising reagent (Section 2.2.5.11). The liner was then rinsed with toluene, dried in a GC oven and the silanised glass wool plug inserted and positioned at approximately the centre of the liner's length. The carrier gas was helium (BOC Gases, ultrahigh purity; 10.9 psi) and 1 µL splitless injections were made with the split closed for

1 min. Unless otherwise specified, all GC/MS methods (drugspe.m, druglle.m, butyl.m, pfpa.m and perform.m) used temperature parameters as follows:-

injector port	250°C (except pfpa.m =260°C)	
transfer line	290°C	
oven -initial	40°C	hold time = 2 min
-final	300°C	hold time = 6 min
temp. rate	10°C/min	

2.4.3 SIM Acquisition

2.4.3.1 *Drug/Drug Derivative Identification and Quantitation*

Two *m/z* ions were selected for their abundance and uniqueness from the full-scan mass spectra obtained for an underivatized or derivatised drug. The more characteristic of the two *m/z* ions selected became the target *m/z* ion and the other, the qualifier *m/z* ion. The ratio of the abundance of qualifier to target *m/z* ion was calculated ($Qualifier_{peak\ area}/Target_{peak\ area}$).

During quantitative drug and drug derivative experiments, acquisition parameters were set to monitor the target *m/z* ions of drugs included in the current working drug solution (Section 2.2.5.14). The time windows set to monitor the *m/z* ions were determined by the retention times of the drug or drug derivative and no more than two drugs were monitored in a time window. Ion dwell times were either 60 or 80 ms.

2.4.3.2 *GC Methods for Comprehensive Drug Screening*

Four methods were created which both acquired and processed data from each of the four blood extracts prepared. Druglle.m and drugspe.m acquire and process data for underivatized extracts from the LLE and SPE techniques respectively. Butyl.m and pfpa.m acquire and process data from the butylated and pentafluoropropionylated extracts respectively. Groups of target *m/z* ion pairs were monitored over specified time periods for groups of closely eluting drugs. Each time period was bracketed by the actual or predicted elution time of consecutive HC markers. In this way new drugs could be easily incorporated in any method once its RI in relation to a HC marker was determined (Appendix A). Similarly, the methods could be easily transported between instruments and laboratories once the retention times of the HC markers were determined on each instrument. Tables 2.1 - 2.4 list the pairs of consecutive *n*-alkanes which bracket a time window, the drug target *m/z* ions and dwell times for each GC acquisition method. The HC marker target *m/z* ion 71.05 was monitored continuously throughout the run time. Dwell time was set to ensure at least 3.0 cycles/s unless the number of *m/z* ions monitored at any one time exceeded 15. In that instance, the number of cycles/s did not fall below 2.44 for any group.

Chromatographic performance was monitored prior to and during every sequence to ensure optimum performance. Perform.m (Table 2.5) was a GC acquisition method only and monitored *m/z* ions for drugs included in the 'performance test mix' of drugs (Section 2.2.5.10). There was no data processing element to this method.

2.5 Comprehensive Drug Screening General Procedure

Despite the chemical diversity of the target drugs a comprehensive drug screen is possible due a combination of extraction procedures and chromatographic manipulation through drug derivatisation. A schematic of the screening procedure is shown in Figure 2.1, and the individual procedures detailed in Figures 2.2 to 2.5. The resultant extracts prepared in Figure 2.2 step 7 ('L'), Figure 2.3 step 14 ('S'), Figure 2.4 step 3 ('B') and Figure 2.5 step 4 ('P') were ready for GC injection.

Although the LLE and SPE procedures could be performed separately for other applications, the developed screening procedure required that they be performed in conjunction. To complete all extraction steps took up to 5 h. Derivatisation steps required a further 2¼ h. The first underivatized blood extracts were ready for GC analysis approximately 5½ h after the procedure was begun. The rate-limiting step was the SPE. Consequently, this screening procedure was performed progressively through to completion without interruption to ensure the first samples were injected by the end of the day. No more than six blood sample (24 GC extracts) were run in a batch as some pentafluoropropionyl drug derivatives were known to deteriorate with time and their stability had only been validated over a 24 h period (Section 5.3.2.4*i*).

2.6 Analysis of Drugs

2.6.1 Chromatographic Data

Chromatographic data was obtained with regard to peak shape, injector carry-over, non-linearity and column overload. Any evidence of decomposition products, of column-priming, or of poor sensitivity and lack of reproducibility were noted for each drug and drug derivative.

Each working drug solution or derivatised working drug solution was diluted 1:1 with the HC marker solution (Section 2.2.5.5) for all GC analyses. These solutions were chromatographed in duplicate and the average of the 'peak area ratios' obtained. The peak area ratio was obtained from the peak areas of the drug or drug derivative and nearest HC marker (peak area ratio = $Drug_{peak\ area}/HC_{peak\ area}$). This ratio eliminated factors which effect absolute peak area from run to run such as variation in injected volume and injector temperature discrimination.

Linear regression equations were determined with four to eleven different working drug solution concentrations. Linear regression equations determined on the narrow bore column were calculated with three to six different concentrations. Linear regression equations determined on the wider bore column were calculated with four to eleven different concentrations. Each different drug concentration and a blank were prepared at the same time and seated in the sample rack of the autosampler awaiting analysis. The different drug amounts were prepared as described in Section 2.2.5.14 except where detection of the drug at the higher amounts was poor. In those cases, the drug amounts prepared were 2 to 3 fold greater than described in Section 2.2.5.14. The approximate 20 ng amount was always the first sample injected. The largest drug amount (approximately 50 ng) was then injected followed consecutively

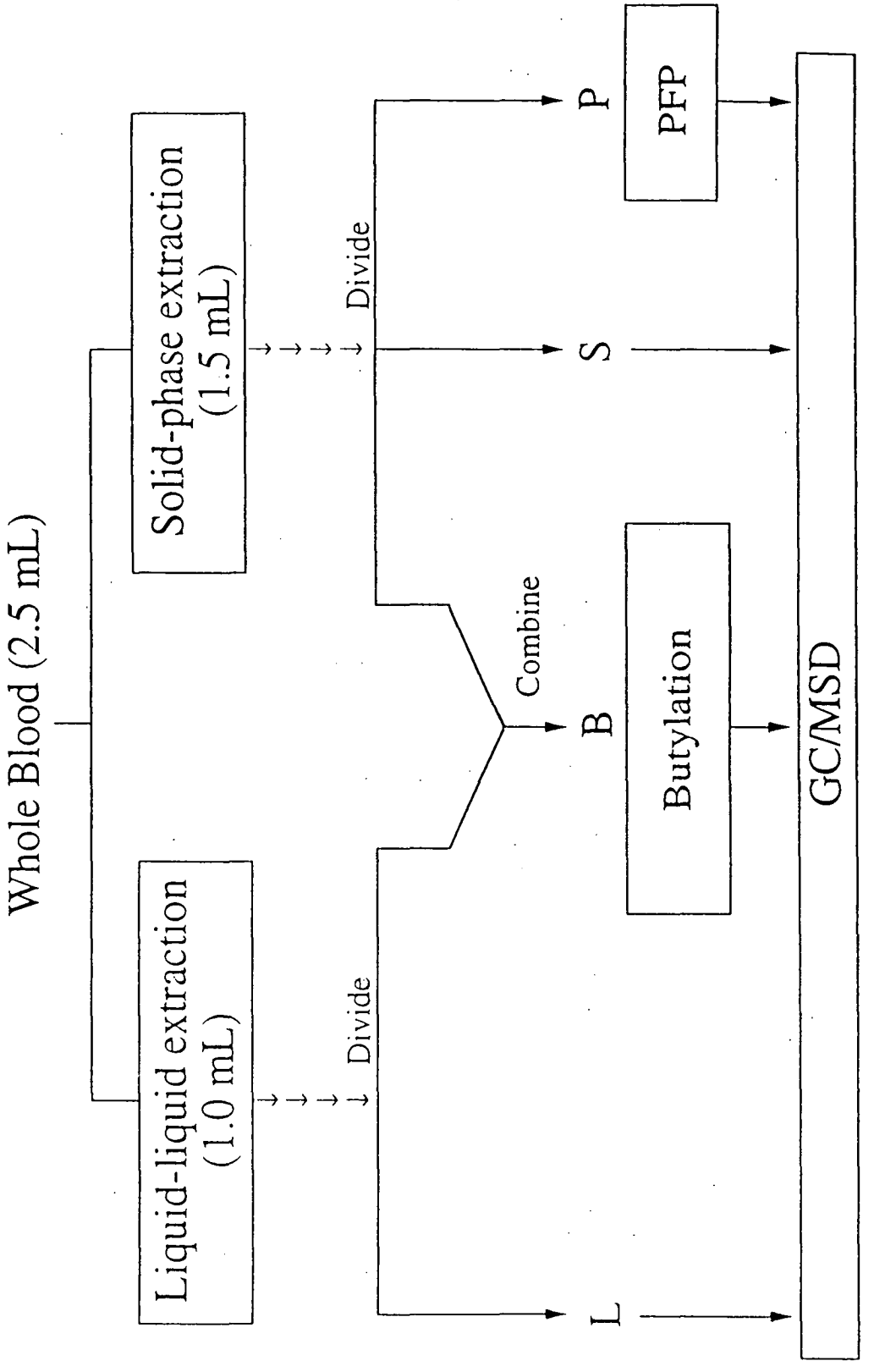


Figure 2.1: Flow diagram of the major steps involved in the comprehensive drug screening procedure.

Figure 2.2: Liquid-liquid extraction procedure.

1.		1.0 mL whole blood into silanised glass tube.
2.	sample pH adjustment	+ 0.5 mL saturated ammonium chloride. Vortexed briefly.
3.	sample extraction	+ 5.0 mL ethyl acetate. Extracted on rotary mixer; 40 rpm for 15 min. Centrifuged; 2000 rpm; 5 min. Retained supernatant in a second silanised glass tube. Sonicated 'plug' for 5 min and re-extracted with a 2nd aliquot of ethyl acetate. Centrifuged; 2000 rpm; 5 min. Combined 2nd supernatant with 1st and reduced volume of combined supernatant to approximately 0.5 mL under nitrogen.
4.	hexane clean-up	Transfered remaining supernatant to 2 mL glass reaction vial labelled 'L' and evaporated to dryness. + 50 µL dried acetonitrile to dried residue. + 0.5 mL hexane saturated with acetonitrile. Capped and agitated vigorously for 20 s. Centrifuged briefly; 2000 rpm. Discarded hexane layer above acetonitrile droplet being careful to discard none of the acetonitrile. Repeated hexane clean-up.
5.		+ 100 µL of hydrocarbon marker solution; 25 µg/mL. + approximately 0.5 mL of chloroform (to increase total volume).
6.	division into 2 identical extracts	To a second reaction vial labelled 'B', transfered approximately half the contents of the original vial such that each contained an approximately equal volume by eye.
7.		Removed organic phase under nitrogen from 'L' extract. + 50 µL chloroform GC injection solvent → GC/MS analysis (refer Section 2.6.2).
8		'B' extract - retained (refer Figure 2.3; step 13).

Figure 2.3: Solid-phase extraction procedure.

1.		1.5 mL whole blood in silanised glass tube. Sonicated; 15 min.
2.	pretreatment of blood	+ 6 mL of 0.1 M phosphate buffer. Mixed briefly by inversion. Centrifuged; 2000 rpm for 15 min.

NB. Vacuum was not applied in the following procedure unless specified.

3.	cartridge conditioning	Passed ≈ 2.5 mL methanol through cartridge. Passed ≈ 2.5 mL 0.1 M phosphate buffer through cartridge being careful that the reservoir did not run completely dry.
4.	sample application	Passed pretreated blood through conditioned cartridge. Pulled last remnants of blood from cartridge under a light vacuum.
5.	cartridge wash	Passed 1.0 mL deionised water through cartridge. Removed last remnants of water under weak vacuum.
6.	pH adjustment	Passed 0.5 mL 0.01 M acetic acid through cartridge. (alters pH conditions for elution of acidic/neutral drugs).
7.	cartridge drying	Removed residual water from the sorbent under strong vacuum; 4 min. + 50 µL dried methanol. Removed residual methanol/water from the sorbent under strong vacuum; 1 min.
8.		Dried needle from each port on the Alltech manifold with tissue and inserted silanised tubes to retain all subsequent eluants.
9.	elution of A/N drugs	+ 4 mL acetone:chloroform (1:1) applied in two 2 mL aliquots.
10.	pH adjusted & elution of B/N drugs	+ 4 mL freshly prepared 5% ammonia in dichloro-methane:isopropanol (80:20) applied in two 2 mL aliquots.
		Discarded cartridges.

Figure 2.3 continued on following page.

Figure 2.3: continued.

11.		To the combined eluants, 150 µL of HC Marker solution was added. Reduced the volume of the combined eluant to ≈2 mL.
12.	division into 3 identical extracts	Transfered to three reaction vials labelled 'S', 'P' and 'B', approximately one third of the remaining eluant such that each contains an approximately equal volume by eye.
13.		Combined contents of 'B' vial with that of 'B' retained in Figure 2.2; step 8 (refer Figure 2.1).
14.		Removed organic phase completely from 'S' extract under nitrogen. + 50 µL chloroform GC injection solvent → GC/MS analysis (refer Section 2.6.2).
15.		Under nitrogen removed contents of vials 'B' and 'P'

Proceed to Figures 2.4 and 2.5 for derivatization of 'B' and 'P' extracts, respectively.

Figure 2.4: Butylation procedure.

		Dried residue contained in reaction vial 'B' (refer Figure 2.3 steps 13 and 15).
1.	reaction medium	+ 20 µL dimethylformamide. + 70 µL TMAH. Vortexed briefly. + 100 µL iodobutane. Vortexed briefly.
2.	reaction conditions	Reacted for 2 h at room temperature vortexing periodically Reacted at 50 °C; 15 min.
3.		Removed reaction mixture under nitrogen. + 50 µL chloroform GC injection solvent → GC/MS analysis (refer Section 2.6.2).

Figure 2.5: Pentafluoropropionylation procedure.

Dried residue contained in reaction vial 'P' (refer Figure 2.3 steps 12 and 15).

1. azeotrope formation
   + 50 µL dried acetonitrile.
   Vortexed briefly.
   Evaporated to near dryness under nitrogen.
   + 50 µL dried acetonitrile.
   Vortexed and evaporated to near dryness under nitrogen.

2. reaction medium
   + 50 µL dried acetonitrile.
   + 50 µL pentafluoropropionic anhydride (PFPA).

3. reaction conditions
   Reacted at 80 °C; 60 min.

4. Removed reaction mixture completely under nitrogen.
   + 50 µL ethyl acetate GC injection solvent → GC/MS analysis (refer Section 2.6.2).

by the smallest amount (blank) to the largest remaining amounts (approximately 4 ng). In this way, potential carry-over from higher drug amounts to lower amounts was avoided except in one instance which was deliberately used to identify carry-over potential associated with each drug.

The retention indices of drug and drug derivatives were calculated from approximately 20 ng/μL working drug solutions and hydrocarbon interpolation was based on HC markers chromatographed concurrently (Appendix A.3.2). Derivatisation reproducibility experiments used drug concentrations of approximately 4 μg/mL. Replicates were prepared on the same day to determine the 'within-day' variations and the 'day-to-day' variations were determined on the following day using the same drug solution which had been stored in the dark at less than -18 °C. Derivatisation stability test used drug concentrations of approximately 20 ng/μL. The GC peak areas of test derivatives were compared with those obtained from a control which was derivatised and immediately analysed (time = 0 h). Following analysis, the control sample remained seated in the rack of the autosampler at room temperature for subsequent analyses after 11 h, and after 16–21 h.

2.6.2 Comprehensive Drug Screening

Four GC methods were created for the acquisition and data processing of each of the extracts as follows:- 'L' extracts were run under druglle.m; 'S' extract were run under drugspe.m; 'B' extracts were run under butyl.m; 'P' extracts were run under pfpa.m.

A permanent screening sequence (macro.s) was created requiring the operator to enter only the identifying number for each blood specimen. All 'S' extracts were chromatographed followed by all 'L', all 'B' and all 'P' extracts.

Prior to each sequence run, the injector liner was replaced by a new silanised injector liner and glass wool (Section 2.4) and a performance check of the chromatographic system carried out using the 'performance test mix' (Section 2.2.5.10).

Figure 2.6 shows a typical acceptable chromatogram of the 'performance test mix'. Drug chromatographic performance was monitored over the entire temperature range of the GC program incorporating early and late eluting drugs. Unacceptable chromatographic performance was indicated by the following.

1. Peaks such as cotinine and phenobarbitone began to tail badly and had a reduced peak height.
2. Temazepam peak height may have been absent or reduced relative to HC peaks.
3. Nordiazepam did not resolve well from disopyramide.
4. Following an initial priming injection, fenfluramine peak height did not remain constant and greater than its nearest HC marker.
5. High boilers (verapamil, thioridazine, diltiazem, prochlorperazine) became broader and had reduced peak heights.

The sequence was not begun unless temazepam could be detected.

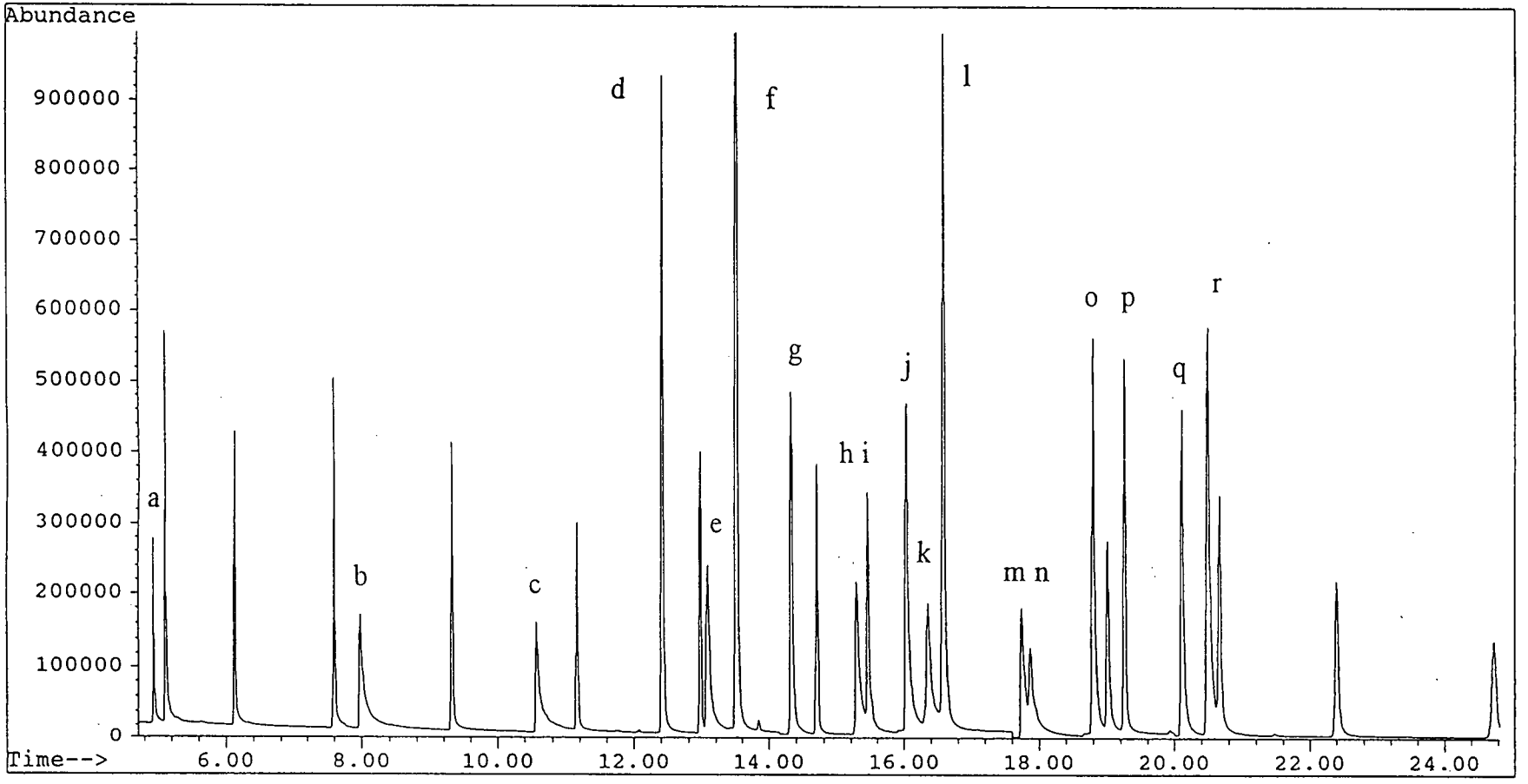


Figure 2.6: SIM generated chromatograms of the performance test mix of drugs and *n*-alkanes (Section 2.2.5.10); fenfluramine (a), cotinine (b), phenobarbitone (c), methadone (d), moclobemide (e), promethazine (f), codeine (g), desmethyldiazepam (h), disopyramide (i), temazepam (j), metoclopramide (k), felodipine (l), quinine (m), prednisolone (n), prochlorperazine (o), diltiazem (p), thioridazine (q) and verapamil (r). The *n*-alkanes are not labelled.

Chromatographic performance was always unacceptable following analysis of the PFP drug derivatives. In general, chromatography could be improved to an acceptable level with the replacement of the injector liner and glass wool. At some stage, however, chromatography could be improved only with the replacement of the pre-column. Underivatised drugs were not analysed following analysis of PFP drug derivatives unless the injector liner and glass wool had been replaced.

2.7. Automated Drug Search and Report

A macro was incorporated in each GC method to automate data analysis of multi-peak chromatograms. Each macro referred to information for each target drug listed in the *drugs.txt* file for that macro (Section 8.3.1). For each drug, the *drugs.txt* file listed the drug name, retention index, target *m/z* ion pair, the alternate extract in which the drug should be identified for confirmation of a macro 'hit', the HC marker used for quantitation (1 of 14), the C and M constants for each linear regression equation, and expected drug recovery. Tables 2.6 to 2.9 reproduce the *drugs.txt* files from druglle.m, drugspe.m, butyl.m and pfpa.m respectively, and list the specific parameters for each drug. Chapter 8 provides greater detail of macro operation.

Chapter 3.

CHROMATOGRAPHY OF DRUGS

3.1 Introduction

The development of an automated broad drug screening procedure in whole blood by benchtop GC/MS required investigation of two areas. One was the isolation and recovery of drugs from the blood matrix (Chapter 7) and the other was drug identification through GC/MS analysis. Drug recovery (extraction techniques, individual drug recovery and reproducibility of extraction) could not be explored without the certainty that each drug investigated could be reproducibly chromatographed and detected at concentrations equivalent to expected therapeutic blood levels.

The following chapters (Chapter 3–5) examine the chromatographic properties of drugs and drug derivatives on a single chromatographic system. The primary aim of these experiments was to generate data which would define the limitations of the instrument as an analytical tool for a large group of structurally-unrelated drugs which possess a variety of different functional groups. By establishing the chromatographic properties of different drugs and drug derivatives, it would then be possible to select target drugs (Table 1.2) for experiments which investigate a comprehensive drug extraction strategy, and eliminate those drugs for which the instrument and/or conditions of analysis were inappropriate (Chapter 6).

In addition to the primary aim, by obtaining chromatographic information for a wide range of drugs and drug derivatives across different drug groups, a database can be established which records the observed behaviour of these analytes on a non-polar chromatographic system (eg. RI, peak shape, linearity over a defined concentration range, limit of instrument detection). These data would allow both individual drugs and drug groups to be compared and comparisons could be made between:-

1. the observed chromatographic behaviour of both underivatised and derivatised drugs;
2. the suitability of different drugs and drug groups to participate in two different derivatisation reactions (Chapter 4; alkylation and Chapter 5; acylation);
3. the effectiveness of the selected derivatisation techniques to produce derivatives from a variety of drugs which have improved chromatographic properties from those of the underivatised drugs; and
4. the reproducibility of formation of different derivatives and their stability with both time and chromatographic temperatures.

The greater the peak area of an analyte reaching the detector at a given concentration, the greater the instrument sensitivity to that analyte. The peak area or chromatographic response for any drug would be greatest when all the injected drug passes unchanged through the separating column to the detector in a narrow focussed band. During the study of drug chromatography, differences in drug retention index (RI) and chromatographic response were investigated (Appendices A and B)

following the manipulation of some chromatographic parameters (eg. column-related variables such as internal diameter, phase ratio, gas flow and connectors joining precolumn and separating column; injector-related variables such as injector temperature, and on-column and splitless injection). It is shown in Appendix B that changes in some chromatographic variables significantly altered the chromatographic response of drug analytes while the response of n-alkanes was largely unaffected. Consequently, the chromatographic conditions which produced the maximum response for the majority of drugs were investigated (Chapters 3–5).

Underivatised drug chromatography was studied following splitless injection using both a narrow bore (0.22 mm i.d) and wider bore (0.32 mm i.d) column. Both qualitative and quantitative aspects of individual drug chromatography were explored. It was hoped that in maximising the chromatographic response of these drugs, detection at concentrations equivalent to therapeutic blood levels and below would be achieved.

3.2 Methods

Refer to "General Procedure" (Chapter 2). In particular, for HC marker solution refer to Sections 2.2.5.5 and 2.2.5.6; for reference drug solutions refer to Section 2.2.5.14; for instrumentation refer to Section 2.4.1; and for SIM acquisition parameters refer to Section 2.4.3.1. Refer to Sections 2.4.2 and 3.2.1 for gas chromatographic parameters with the wider bore and narrow bore columns respectively. Qualitative and quantitative chromatographic data for drugs were obtained using the method described in Section 2.6.1 unless otherwise specified.

3.3 Results

3.3.1 Drug Chromatography on the Narrow Bore Column

The chromatographic separation of a mixture of drugs on the capillary column occurs as a result of the distribution of the drug molecules between the gas and stationary phases.[1] Movement of the drugs through the column occurs as a result of repeated sorption/desorption along the stationary phase.[1] Separation of the drug mixture occurs because different drugs spend different amounts of time in the stationary phase and, consequently, exit the column at different times.[1] The chromatographic characteristics of drugs are independent of their pharmacological action and are governed by a number of factors including their relative 'polarities'. In the chromatographic literature, it is apparent that 'polarity' is a term which could loosely describe the potential of an analyte to participate in additional column-related interactions during its elution and which affect its partitioning between the gas and liquid phases. Consequently, it might be expected that the 'chromatographic polarity' of a drug is influenced by the entire chemical structure of the drug (atomic composition and the arrangement of the individual atoms in relation to others) but more specifically by the presence of potentially reactive functional groups within that structure.

Table 3.1 shows chromatographic data for drug standards chromatographed on the

narrow bore column following splitless injection at an injector temperature of 245°C. Where possible, drugs in Table 3.1 are categorised under headings which reflect a common chemical structure ('structurally-related drugs' - eg. 'xanthine', 'phenothiazine'). These drugs are structural analogues and differ only marginally from others within a group, for example by the presence, absence or rearrangements of side-chains. Some drug groupings do describe a pharmacological action (eg. tricyclic anti-depressants; anti-diabetic and ß-blocking agents). In these cases, the pharmacological action remains unimportant to drug chromatography and 'polarity', but it reflects a common chemical structure which unite these drugs as structural analogues (eg. Figure 5.3; ß-blocking agents). The chemical names of these groups are not as easily related to drug structure as others used in Table 3.1, and for convenience, the pharmacological action identifies these drugs. It was not valid to suspect, however, that all drugs with a common pharmacological action also possessed a common chemical structure. For example, the phenylethylamine derivatives are all CNS stimulants, as is cocaine. Cocaine, however, is chemically dissimilar to the phenylethylamine derivatives and, therefore, its chromatographic properties are likely to be very different. Figure 3.1 illustrates the chemical structures of representative drugs described by each of group headings used in Table 3.1 for structurally-related drugs. Figure 5.3 shows ß-blocking agents.

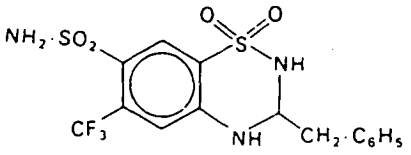
As the chemical structures of drugs are diverse, it was not possible to categorise many of the drugs selected for investigation under a 'structurally-related drug' group. Consequently, drugs which were otherwise structurally-unrelated were grouped with others which possessed a common reactive functional group ('structurally-unrelated drugs' - eg. a carboxylic acid or 1° amine group). Where a drug possessed two or more different functional groups, it was grouped with other polyfunctional drugs.

Some structurally-unrelated drugs had no reactive functional groups and, consequently, both their reactivity and expected 'chromatographic polarity' were reduced compared with 1° amines or phenols for example. These apparently 'non-polar' drugs were grouped together. Some drugs within this large group of non-polar drugs were further united by common nitrogen-containing heterocyclic structures. Although their full chemical structures were not similar enough to be classed as structural analogues, the nitrogen-containing cyclic compounds shown in Figure 3.2, and their derivatives, formed part of the chemical structures of these drugs and, consequently, they were categorised as 'nitrogen heterocyclic drugs'. It should be noted that some of the structurally-related drug groups also describe apparently 'non-polar' drugs (eg. phenothiazine derivatives and anti-histamines have no reactive functional groups). The drug categories used in Table 3.1 are the same as those used in all tables from Chapters 3–5.

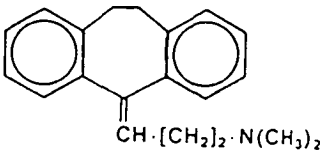
Mixed drug solutions were analysed by low resolution EI full-scan mass spectrometry. Where a peak was chromatographed, it was identified from its full-scan mass spectra and target *m/z* ions were selected for each drug. Drug RI and ion abundance ratios were determined (Section 3.2).

Data in Table 3.1 clearly show that not all drugs investigated could be chromatographed under the selected GC conditions (-). Diuretic agents, the majority

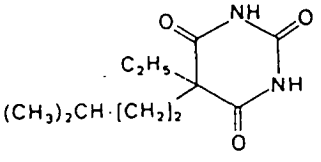
Diuretic agent
(bendrofluazide)



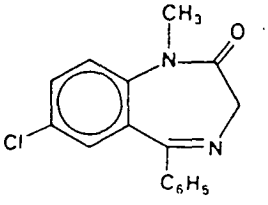
Tricyclic anti-depressant agent
(amitriptyline)



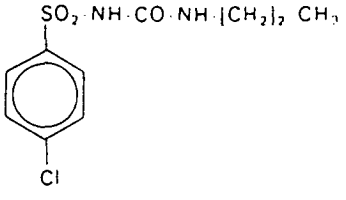
Barbiturate
(amylobarbitone)



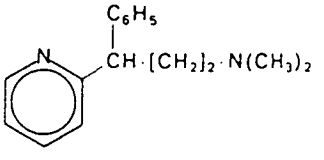
Benzodiazepine
(diazepam)



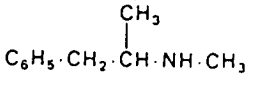
Anti-diabetic agent
(chlorpropamide)



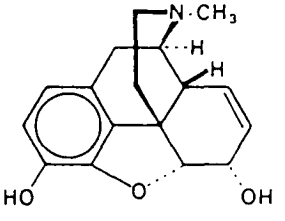
Anti-histamine
(pheniramine)



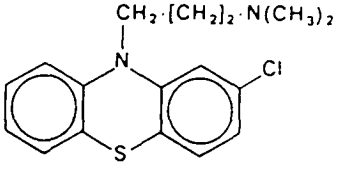
Phenylethylamine derivative
(methylamphetamine)



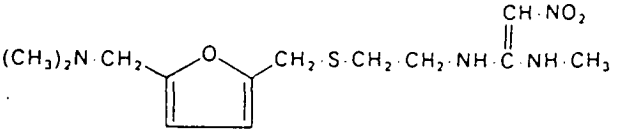
Opiate
(morphine)



Phenothiazine derivative
(chlorpromazine)



H_2-receptor antagonist
(ranitidine)



Xanthine derivative
(caffeine)

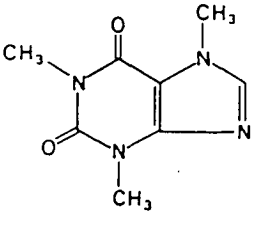
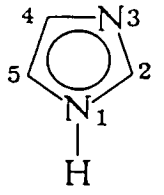
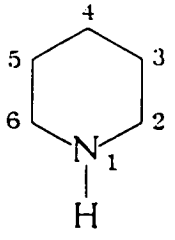


Figure 3.1: The chemical structures of representative drugs described by the group headings used in Table 3.1 for structurally-related drugs.[2]

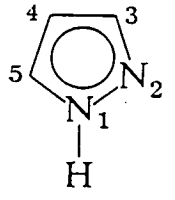
Imidazole



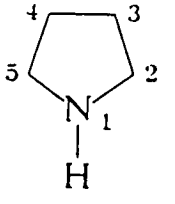
Piperidine



Pyrazole



Pyrrolidine



Quinoline

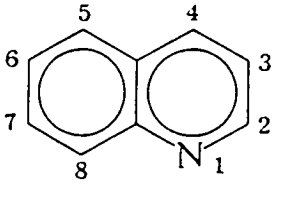


Figure 3.2: Nitrogen-containing heterocyclic compounds (Section 3.3.1).[3]

of drugs with carboxylic acid functions and polyfunctional drugs did not chromatograph under the selected conditions. The anti-diabetic agents and H_2-receptor antagonists were either not chromatographed or chromatographed only as GC artefacts of the drug (Section 5.3.2.2). Hyoscine and hyoscyamine were chromatographed as GC artefacts at both the set injector temperature and a lower injector temperature of 235°C. In contrast, carbamazepine was detected at the lower injector temperature while the artefact was chromatographed at an injector temperature of 245°C.

A GC peak which corresponded to the drug or a GC artefact of the drug was detected for the majority of drugs from all other groupings.

Table 3.2 shows quantitative and qualitative chromatographic data for those drug standards able to be chromatographed on the narrow bore column following splitless injection. As described in Section 2.6.1, each data point employed in determining linear regression equations was a peak area ratio (the peak area obtained for the target *m/z* ion for that drug divided by the peak area of *m/z* 71.05 for the closest eluting *n*-alkane in a GC run). Table 3.2 lists both the target *m/z* ion monitored and the reference HC marker for individual drugs from which the peak area ratios were calculated.

Some drugs were found to decompose at an injector temperature of 245°C (Table 5.6 and Appendix B;Table B.3). As maximum chromatographic response was to be investigated, these temperature-sensitive drugs were chromatographed at a lower injector temperature of 235°C as indicated in Table 3.2.

Table 3.2 shows linearity data over a defined concentration range for those drug standards which were able to be chromatographed. The concentration range is defined by the 'highest' and 'lowest' drug amounts. The 'lowest' amount injected refers to the smallest amount of drug injected which produced a peak that could be integrated. One hundred- to 1000-fold differences in amounts injected were chromatographed for the majority of drugs.

Table 3.2 shows information for most drugs with regard to shape of the peak chromatographed (A), carry over from the injector to a following blank sample (B), thermal decomposition of that peak (C) and, where known, the amount injected which caused column overload [x]. The linear regression equation shown for each drug described the line of best fit for the $\log_{10}$ of the raw data points as follows:-

$$\log y = C + M.\log x$$

where y = peak area ratio
x = amount of drug injected
C and M are constants in the equation describing transformed data.

Consequently, the linear regression equation for the log transformed data becomes

$$Y = C + MX$$

where $Y = \log y$
$X = \log x$.

The equation for each drug was fitted to log transformed data points (from 3 to 6) over a concentration range between, and including, the 'lowest' and 'highest' amounts injected as listed in Table 3.2. The log transformed rather than raw data was employed for two reasons.

1. It removed the bias towards large y values (nanogram versus picogram drug amounts) and fitted the line of best fit to equally weighted data points over the entire injected range.
2. It allowed data for all drugs to be described by a simple linear equation in contrast to untransformed data which would be best described by linear equations for some drugs and more complicated non-linear equations for others.

When $r^2 = 1.00$, both the log-transformed and untransformed data would be best described by the given equation (Table 3.2) and anti-log of that equation respectively, over the entire concentration range.

The relationship between log-transformed and raw data can be determined as follows:-

$$Y = C + MX$$

taking the antilog of the equation $10^Y = 10^{C + MX}$

then $10^Y = 10^C.10^{MX}$

replacing Y and X with log y and log x $y = 10^C.x^M$

Consequently, a power equation best describes the untransformed data, and this equation is linear when M = 1.0. When M is greater than or less than 1.0, the equation describes a curve rather than a straight line. Clearly, the greater the value of either C or M, the greater the chromatographic response (y) to an increase in amount of drug injected (x).

If the amount injected (x) was doubled from 1 to 2 then, when M = 2, the increase in response (y) would be four times greater than if M = 1.

where M = 2 then $y = 10^C.x^2$

where x = 1 then $y = 10^C.(1)$

where x = 2 then $y = 10^C.(4)$

When M is the same, or very similar, for two drugs, the C value describes the magnitude of the change in detector response with a change in the drug amount injected and, the more positive the C value for a particular drug, the greater the detector response/sensitivity to the change. Consequently, C is useful for comparison of drugs only when M is the same, but M allows comparison of all drugs.

Table 3.2 gives the factor (10^M) by which chromatographic response would increase following an increase in the amount injected from 1 to 10 (pg or ng) for each drug standard. Consequently, for drug standards with regression equations which were approximately linear, this factor would be approximately 10.

where M = 1 then $y = 10^C.x$

for 1 pg or ng drug injected $y = 10^C.(1)$

for 10 pg or ng drug injected $y = 10^C.(10)$

For drug standards with power regression equations ($M \neq 1$), the 10^M factor would be greater than or less than 10. The 10^M factor is listed in Table 3.2 as it is simpler to appreciate the departure from linearity demonstrated by a ten-fold increase in the amount injected than by a difference between absolute M values.

For example where M = 1.3, the 10^M value = 19.9

where M = 1.5, the 10^M value = 31.2

Therefore, the 10^M factor is a good indicator or drug linearity over the concentration range tested.

Data in Table 3.2 indicate that the chromatographic properties of drug standards within a group were not consistent even among drugs with very similar chemical structures. For example, the individual drugs within the benzodiazepine, phenylethylamine or phenothiazine groups differed in their chromatographic properties despite their structural similarity. Individual drugs from these groups exhibited the following range of properties when compared with other drugs from the same group.

1. Peak shapes ranged from Gaussian to excessively tailing.
2. Either linear or power regression equations described the untransformed data.
3. Drugs were detected at injected amounts which ranged from picogram to nanogram quantities.
4. For some drugs, there was no evidence of carry-over of the sample to the following blank injection while others showed carry-over in both consecutively run blank injections.

Thermal decomposition products were detected for some phenylethylamine derivatives and not others despite a low injector temperature of 235°C. Similarly, the reproducibility of duplicate peak areas was so poor for methdilazine (phenothiazine group) that no quantitative data was obtained while those of trimeprazine were very reproducible and this drug was detected at low picogram amounts.

Structurally-related xanthine derivatives, theophylline and caffeine (methyl-theophylline) possessed very different chromatographic properties. Theophylline decomposed on injection and chromatographed with a tailing peak shape. Caffeine, however, was detected at picogram levels, showed no evidence of thermal decomposition and was linear over the concentration range tested. Similar differences in the chromatographic properties of other drugs and their desmethyl metabolites were also observed (eg. amitriptyline and nortriptyline; imipramine and desipramine; methylphenobarbitone and phenobarbitone; diazepam and desmethyldiazepam).

Data in Table 3.2 indicate that the structurally-related tricyclic anti-depressant agents chromatographed with Gaussian to slightly tailing peak shapes. These drugs were detected at picogram levels except nortriptyline, protriptyline and desipramine and its artefact which were detected at nanogram levels. The the data points of the anti-depressant drugs were fit by linear regression equations which were all relatively linear ($10^M < 26$) except for amitriptyline and those detected at nanogram levels.

The barbiturates were chromatographed with Gaussian peak shapes and were approximately linear over the concentration range tested except phenobarbitone and

primidone. Decomposition of the primidone peak was evident and the raw data points of phenobarbitone were fitted by a power equation (10^M approximately 2).

The peak shapes of the morphine derivatives were generally poor except for that of dextromethorphan which was also detected at the lowest injected amount (< 70 pg).

From data in Table 3.1 it was observed that one of the ß-blocking agents (metoprolol) was not able to be chromatographed. Data in Table 3.2 indicate that although the remaining two ß-blocking agents were chromatographed, decomposition products were detected.

Structurally similar anti-histamines all chromatographed with Gaussian peak shapes but varied in other chromatographic properties. For example, doxylamine and pheniramine were detected at injected amounts ten times greater than the other drugs. Similarly, linearity over the concentration range tested was poor for pheniramine (10^M = 107) while that of the other drugs was relatively better (10^M = 12 - 25).

There was little consistency in the chromatographic properties of individual drugs within groups which described drugs with common functional groups but which were otherwise structurally unrelated. The only trend apparent was in the 1° amine and carboxylic acid groups in which all drugs investigated were poorly chromatographed. All other drugs showed the same range of different chromatographic properties (peak shape, lowest amount detected, linearity over the concentration range tested, carry-over and thermal stability) as reported for the structurally-related drugs.

3.3.2 Drug Chromatography on the Wider Bore Column

Drug standards were chromatographed on the wider bore column following splitless injection at an injector temperature of 250°C. All drugs were investigated at this injector temperature despite their thermal instability. Consequently, no GC peak was detected for carbimazole (Appendix B; Table B.3) or phenylpropanolamine. The ranges (indicated by the upper values) within which the detection limits of amphetamine and methylamphetamine fell increased, from 0.2 ng and 0.24 ng respectively, on the narrow bore column to 18 ng and 2.0 ng respectively, on the wider bore column. Similarly, carbamazepine was largely chromatographed as a GC artefact following injection at 250°C and the range within which its detection limit fell increased from 0.30 - 0.76 ng on the narrow bore column to 5.7 - 28.6 ng on the wider bore column.

During the investigation of the effect of altered chromatographic variables on drug chromatography (Appendices A and B), the following was indicated.

1. The detection limit for 93% of the drugs chromatographed on the wider bore column was less than or similar to that determined on the narrow bore column with half the drugs in each categrory. For some drugs the detection limit on the wider bore column was one tenth of that determined on the narrow bore column (Appendix B.3.2.1*ii* and Table B.12).
2. The calculated RI of a given drug was lower on the wider bore than the narrow bore column and the difference became greater as carbon number increased (Appendix A.3 and Table A.2).

3. The differences in film thickness and diameter between the narrow and wider bore columns resulted in the narrow bore column having a greater column capacity than the wider bore column. Consequently, for drugs chromatographed on the wider bore column, there was a decrease in the amount of drug injected onto the column which produced an overloaded peak (Section B.3.2.3).
4. The chromatographic response of both thermally sensitive drugs and high boiling drugs was improved when chromatographed on the wider bore compared to the narrow bore column.

The peak shapes chromatographed for some drugs on the wider bore column improved when compared with their chromatography on the narrow bore column. For example, the peak shapes of some drugs improved as follows:- amitriptyline (A3 to A1), desipramine (A3 to A1), dihydrocodeine (A4 to A3), dothiepin (A3 to A1), desmethyldiazepam (A4 to A3), dextromethorphan (A2 to A1), diltiazem (A4 to A1), benztropine (A5 to A3), trimethoprim (A4 to A3) and hyoscine (A3 to A2). In some cases, substantial improvement in chromatography on the wider bore column was observed from that on the narrow bore column (eg oxycodone:- peak absent to A1; benztropine, methdilazine and dextropropoxyphene:- A5 to A1). Pilocarpine and methoxyphenamine, which had each chromatographed as two unresolved peak on the narrow bore column, chromatographed as a single tailing peak on the wider bore column.

The peak shape for other drugs which did not chromatograph well on the narrow bore column were so improved following their chromatography on the wider bore column that qualitative and quantitative chromatographic data was obtained as shown in Tables 3.3 and 3.4 (eg captopril, dextropropoxyphene, diclofenac, mebhydrolin, methdilazine, methoxyphenamine, oxycodone, pilocarpine, primidone). The drug groupings and types of chromatographic data shown in Tables 3.3 and 3.4 are the same as those in Tables 3.1 and 3.2, respectively. Although these drugs were now able to be chromatographed, however, the following was observed.

1. In the majority of cases, linearity over the concentration range tested was poor ($10^M > 20$).
2. The chromatography of some drugs was too poor to obtain linearity data (eg. captopril artefact, theophylline, trimethoprim, quinine and quinidine).
3. Some drugs for which linearity data was obtained showed poor sensitivity; their detection limit was relatively high compared with other drugs chromatographed under the same conditions (eg. hyoscyamine, benzodiazepine metabolites, oxycodone).

The peak shapes chromatographed for some drugs on the wider bore column deteriorated when compared with their chromatography on the narrow bore column as follows:- cotinine (A1 to A3), phenindione (A1 to A4), metoclopramide (A1 to A3). In addition to poor peak shape, decomposition products were detected for MDMA and paracetamol.

The carry over characteristics of some drugs altered on the wider bore column from those observed on the narrow bore column as follows:- ibuprofen (B1 to B2),

methylphenobarbitone (B1 to B2), diclofenac (B4 to B2), sulphinpyrazone (B1 to B4), THC (B1 to B2).

Where there was improvement in either the peak shape or the detection limit of drugs chromatographed on the wider bore column from that observed on the narrow bore column, linearity (10^{M}_{WIDE}) over the concentration range tested also improved although power equations still described the curve of best fit for untransformed data points (Table 3.2; $10 < 10^{M}_{WIDE} < 10^{M}$). It should be noted that, where both 10^{M} and 10^{M}_{WIDE} values are recorded, a narrower concentration range was investigated on the wider bore column (approximately 200 pg to 30 ng) than on the narrow bore column (approximately 50 pg to 50 ng). Data in Table 3.2 indicate that, in general, the majority of anti-depressant agents, barbiturates and anti-histamines (including pheniramine) were relatively linear over the concentration range tested. The non-polar drugs and nitrogen heterocyclic drugs were relatively linear although there were some obvious exceptions (eg. verapamil, clonidine). In contrast, the majority of benzodiazepines, and 1° and 2° amines were described by power equations over the concentration range tested.

Tables 3.3 and 3.4 also show chromatographic data for additional drug standards chromatographed on the wider bore column which had not previously been investigated on the narrow bore column (eg. some benzodiazepines and their metabolites). Data indicate that, for these drugs, power equations best described the curve fitted to untransformed data points except for GC artefacts of dextropropoxyphene and carbamazepine.

3.4 Discussion

The chromatographic characteristics of a wide range of drugs across different drug groups was investigated following splitless injection using both narrow and wider bore columns. The chromatographic information recorded establishes a database of observed behaviour for these analytes on a non-polar chromatographic system (ie. RI, peak shape, linearity over a defined concentration range, limit of instrument detection). Both qualitative and quantitative aspects of individual drug chromatography were reported allowing an evaluation of the chromatographic performance of a large number of structurally dissimilar drugs under set conditions. The chromatographic performance of different drugs can be compared using criteria such as peak shape, linearity over a defined concentration range (10^{M}), 'carry-over' potential, thermal stability and instrument sensitivity (indicated by drug detection limit).

The chromatographic response for any drug would be maximised when all the injected drug passes unchanged through the separating column to the detector in a narrow focussed band (Section 3.1). Consequently, chromatographic response is influenced by the following considerations.

1. If the drug decomposed either partially or wholly in either the injector or during transit through the column, the amount of drug reaching the detector would be reduced. Several drugs showed evidence of decomposition products which were either completely or partially resolved from the drug peak (Tables 3.2 and 3.4).

Drug instability at elevated temperatures is discussed in Section 5.4.2.1 using the example of PFP-drug derivatives.

2. If the same amount of two different drugs was injected onto the column and one drug was optimally re-focussed on the front of the column and underwent minimal longitudinal diffusion during elution (Appendix B.4.1.2), and the other was poorly refocussed and/or experienced relatively more longitudinal diffusion then, although each drug would reach the detector, one would elute as a narrow band and the other as a band spread over a relatively wider area. Although the detector would indicate the same total amount for each drug, the signal representing the drug eluting in a narrow band would be high and of short duration while that of the drug eluting in a relatively broader band would be lower and of longer duration. Consequently, if the same two drugs were chromatographed at a concentration close to the critical threshold of instrument detection, the detector would have greater sensitivity to the drug eluting as a narrow band; this drug would have the greater chromatographic response. The detection limit of a drug, therefore, is likely to be a very good indicator of whether or not a drug chromatographing under the set conditions possessed good chromatographic properties (Table B.12).
3. Peak shapes in Table 3.2 and 3.4 ranged from Gaussian (A1) to excessively tailing (A5). Peak shape provides an indicator of band width on the column. If all molecules of drug were relatively uniformly distributed throughout the band and well focussed, only Gaussian peak shapes would result as the majority of molecules would be distributed towards the centre of the band and fewer and fewer molecules distributed towards its extremes in a 'normal/Gaussian distribution'. Tailing peak shapes imply that individual drug molecules are not normally distributed within the band but that the majority of drug molecules are concentrated in the leading front of the band with the remainder extending from there in diminishing number towards the back of the band. Longitudinal diffusion of the band as it moves along the column does not explain the non-uniform distribution of drug molecules in tailing peaks. However, increasing or decreasing longitudinal diffusion (governed by longer or shorter elution time) either exaggerates or masks the 'tail' in these heterogenous bands.
4. It was stated in Section 3.3 that the chromatographic separation of a mixture of drugs occurred as a result of the partitioning of drugs between the gas and stationary phases and that drug retention was dependent on the amount of time spent in the stationary phase (partition coefficient).[1] Drug retention, however, is further influenced by additional interactions of the drug with the stationary phase - or 'interfacial' adsorption - at both the column support and the gas-liquid interfaces.[1] The column support is not entirely inert and despite careful deactivation, cannot completely eliminate its contribution to drug retention.[1] Similarly, adsorption at the gas-liquid interface is likely to become increasingly significant as the chemical dissimilarity between the non-polar stationary phase (cross-linked methyl silicone) and relatively more 'polar' drugs increase.[1] There are few studies which explore the retention mechanism for open tubular columns with cross-linked stationary phase such as that used in the present experiments, but 'interfacial' adsorption has been shown to affect the reproducibility of retention for columns with non-polar phases of different thicknesses,[1] and was shown to contribute to the retention of polar solutes on poly(dimethylsiloxane)

phases which increased when the phase was cross-linked.[1]

5. If individual drug molecules were partially or completely adsorbed to active sites either in the injector or on the column (column support) following their injection, then either a smaller (reduced absolute peak area) or no, peak would be detected. Similarly, if individual drug molecules interacted excessively with the stationary phase during their elution (partial or complete adsorption at the gas-liquid interface), broadened and/or tailing, or smaller peaks would elute from the column rather than the 'Gaussian' peaks anticipated from a narrow, focussed band of drug molecules. Both conditions would result in a decreased chromatographic response for that drug. Drugs of different 'polarities' interact to differing degrees with both active sites in the chromatographic system and the stationary phase of the column during their elution. These additional interactions are minimal during the elution of non-polar drugs as their movement along the stationary phase is governed largely by their partition coefficient. For more polar drugs, however, the interactions can be significant as their elution is affected not only by their partition coefficients but by 'interfacial' adsorption. Improving the chromatographic characteristics of polar drugs would, therefore, lead to improved chromatographic response and detector sensitivity.

In general, there was an improvement in the chromatography of a drug on the wider bore column from that observed on the narrow bore column. This was reflected by improved peak shapes, a decreased range in which the limit of detection fell and improved linearity over the tested concentration range ($10^{M} > 10^{M}_{WIDE}$). The major factors which improved the chromatographic responses of drugs are discussed in Appendices A.4 (higher phase ratio and faster elution times), B.4.1 (injector-related factors) and B.4.2 (increased gas flow through the column, and exposure to reduced temperature ranges prior to elution). These factors influenced both the amount of drug reaching the detector and the width of the band eluting from the column to the detector, effectively diminishing chromatographic problems associated with polarity for some drugs. The effect of these factors on drug chromatography is demonstrated by the differences observed in the chromatographic behaviour of some individual drugs on the two columns as follows.

1. Thermal instability at an injector temperature of 250°C resulted in poorer chromatographic responses on the wider bore than narrow bore column for amphetamine, methylamphetamine and carbamazepine as reflected by increased detection limits. Similarly, thermal decomposition was complete for carbimazole and phenylpropanolamine which were not able to be chromatographed on the wider bore column. Only carbamazepine produced a GC artefact/decomposition product that was stable enough to be chromatographed and for which chromatographic data was obtained, although decomposition products were apparent for MDMA and paracetamol.
2. The GC artefact of hyoscyamine was chromatographed on both narrow and wider bore columns. However, although hyoscyamine was not chromatographed 'intact' on the narrow bore column, it was apparent on the wider bore column. Higher gas flow through the injector at the time of injection resulted in a briefer exposure of the drug to the high temperatures and, consequently, some intact drug passed on to the wider bore column (Appendix B 3.2.2 and Section 5.4.2.1). The proportion of 'intact' drug passing from the injector was small, however, as indicated by the

relatively high limit of detection for the drug. In addition, hyoscyamine was a relatively polar drug compared with its GC artefact as indicated by the 10^M values (72.4 and 25.1, respectively).

3. Pilocarpine and methoxyphenamine, which had each chromatographed as two unresolved peaks on the narrow bore column, chromatographed as a single tailing peak on the wider bore column. Faster elution times on the wider bore column resulted in poorer resolution between these drugs and their decomposition products giving the appearance of a single tailing peak instead of two partially resolved peaks.

As the chromatographic behaviour of drugs is affected by their apparent 'polarity', chromatography on the wider bore could not significantly alter the chromatographic properties of these polar drugs. It simply tended to mask tailing/poor peak shapes as drugs eluted from the column more rapidly (Appendix A.4). For example, although selected polar drugs were now able to be chromatographed, their linearity over the concentration range tested was still poor. Similarly, the chromatography of other polar drugs was still relatively poor, as reflected by $10^M > 20$, and relatively high detection limits.

In general, the non-polar drugs possessed chromatographic properties which ensured good peak shape, linearity and low detection limits. In contrast, the apparently 'polar' nature of some acidic (eg carboxylic acids and phenols) and basic (eg amines, ß-blocking agents) drugs resulted in the loss of the injected sample through irreversible column adsorption (higher limits of instrument detection) which also affected the injected range over which chromatography was linear (power equations were observed). The poorer chromatographic properties of weakly to strongly polar drugs compared with non-polar drugs resulted from undesirable drug-stationary phase interactions discussed in Sections 5.4.2.2 to 5.4.2.4. Even some of the apparently 'non-polar' drugs listed in Table 3.2 (eg mebhydrolin) possessed residual polarity imparted to them by their total chemical composition which adversely affected their chromatographic performance. Consequently, the observed chromatographic behaviour of a wide range of chemically dissimilar drugs has permitted an estimation and comparison of apparent 'chromatographic polarity' across drug groups.

The effect of drug 'polarity' on drug chromatography was clearly illustrated when a comparison was made of the chromatographic properties of drugs and their desmethyl metabolites. The chemical structures of the metabolites differed from the drugs only by the absence of a methyl group. Reactive functional groups which increased both the reactivity and polarity of the metabolite were revealed when the relatively non-polar drug lost a methyl group. In the absence of the methyl group, poorer peak shapes, chromatographic linearity and detection limits were observed for each metabolite when compared with the drug.

Although a greater chromatographic response was attained for the majority of drugs chromatographed on the wider bore column, clearly the chromatographic characteristics of other acidic and basic drugs require improvement if they are to be incorporated into a broad drug screening procedure.

References

1. "Chromatography Today."
Poole C, Poole S (Eds.) 1st Edition (1991) Elsevier Science Publishing Company, New York; 169-176

2. "Organic Chemistry."
Kemp D S, Vellaccio F; 1980, 817; Worth Publishers Inc. USA

3. "Clarke's Isolation and Identification of Drugs."
Moffat A (Ed.); 1986, 2nd Edition, The Pharmaceutical Press, London

Chapter 4.

ALKYLATION AND CHROMATOGRAPHY OF ALKYL DERIVATIVES.

4.1 Introduction

4.1.1 General

Tables 3.1–3.4 showed qualitative and quantitative chromatographic data for underivatised drugs. These data indicated that drugs with carboxylic acid functional groups were too polar to chromatograph on the non-polar cross-linked methyl silicone stationary phase of either high performance capillary column employed, and confirmed similar findings in the literature.[1,2,3] It was also shown that many weakly acidic drugs (eg phenols) were chromatographed as broad, tailing peaks (rather than with Gaussian peak shapes) and possessed poor linearity characteristics over the concentration range tested, and relatively high detection limits. In Tables 3.2 and 3.4 it was shown that many desmethyl drug metabolites were relatively poorly chromatographed in comparison with the 'methylated' drugs. The chromatography of acidic and basic drugs could be improved if their polar functional groups were shielded during elution to minimise undesirable column interactions which could lead to irreversible column adsorption and asymmetric peak shapes. Chemical derivatisation of potentially reactive functional groups with non-polar molecules is a technique often employed in GC analyses to improve analyte chromatography.[2,3]

The term 'alkylation' covers a variety of techniques in which an acidic proton can be replaced by a non-polar alkyl group.[1,3] Consequently, alkylation is a derivatisation technique particularly suited to analytes possessing acidic functional groups, and its application to acidic drugs was investigated. Following the alkylation of an acidic drug, it might be expected that the resulting alkylated derivative will be less polar than the underivatised drug and, consequently, improved chromatographic performance will result.

The strength of an acid is given by its dissociation constant, usually as the negative $\log_{10}$ or pKa, and describes the ease of dissociation of the acidic proton. The lower the pKa, the stronger the acid and the greater the potential for that drug to be derivatised by an alkylation reaction.

Many alkylation reactions are possible employing different reagents,[1,2,3] however, investigation was limited to drug alkylation using either diazomethane or *n*-butyl iodide. Diazomethane was selected as methyl derivatives are very commonly reported in the literature (eg. mass spectral and RI databases)[2,4] and methylation is rapid. *n*-Butyl iodide was selected as the literature reported that this reagent was able to derivatise a wide range of weakly to strongly acidic drugs, including benzodiazepines.[1,2,5-9] Data in Tables 3.1–3.4 indicated that many benzodiazepines were poorly chromatographed. It was important that these drugs be included in the broad drug screening procedure, however, as they are prevalent

in the community, have a high potential for abuse and are of toxicological significance (Table 1.2).

Developmental work for the alkylation technique was performed on the narrow bore capillary column. The qualitative and quantitative chromatographic characteristics of butyl drug derivatives were then investigated following splitless injection using both the narrow bore (0.22 mm i.d) and wider bore (0.32 mm i.d) columns. The qualitative chromatographic characteristics of methyl drug derivatives, however, were investigated only on the narrow bore column. It was hoped that in maximising the chromatographic response of these polar drugs through derivatisation, detection at concentrations equivalent to therapeutic blood levels and below would be achieved.

4.1.2 Diazomethane

The most commonly used reagent in diazoalkane alkylation reactions is diazomethane, and methyl derivatives are produced. Diazomethane is gaseous, toxic and explosive.[1,2] It can be either bubbled through a solution of the acidic drug, or a solution of the gas in a suitable solvent can be added to the drug. Generally, ethereal solutions of diazomethane are prepared, but these solutions are not very stable and can be stored for only a few weeks at low temperature (-18°C).[2] The reaction does not expose the drug to high temperatures or reaction conditions that can cause decomposition and, provided the choice of solvent is correct, the rate of reaction is usually high.[1] On completion, excess reagent can be removed by evaporation under a stream of nitrogen.

Methylation with diazomethane is represented by the following:

$$R^{-}\text{-}H^{+} + \text{diazomethane} \rightarrow R\text{-}CH_3 + \text{end products} \qquad \text{Equation 4.1}$$

During reaction with diazomethane, unexpected side-reactions and *cis - trans* isomerisation have been reported.[2] The removal of excess diazomethane prior to analysis can minimise some side-reactions, although volatile methyl esters can be simultaneously lost by evaporation.[2]

Following reaction of drugs with diazomethane, the methyl derivatives formed are predictable.[2] The molecular weight increases by 14 or 28 amu (mono- or bis-derivatives respectively). The fragmentation patterns of the EI mass spectra generated for methyl derivatives differ little from those of the underivatised drugs and many commercially available libraries of drug mass spectra include methylated derivatives.

4.1.3 *n*-Butyl Iodide

The reaction mechanisms for the alkylation of acidic compounds by alkyl halides and have been reviewed.[1,2,5] Alkylation reactions employing alkyl halides are bimolecular nucleophilic substitutions (S_N2) at a saturated carbon.[1,5] A nucleophile (the deprotonated acid) displaces a leaving group (the halide) from the substrate (the alkyl halide). The displacement occurs provided that:

1. both the nucleophile and leaving group are anionic or neutral bases with

unshared electron pairs, and
2. the nucleophilicity of the deprotonated acid is greater than that of the halide.[1,5]

The deprotonation of the acidic drugs can be catalysed by the addition of a basic agent, for example tetramethylammonium hydroxide (TMAH). In this alkaline environment the acidic drug is deprotonated by the following instantaneous reaction and an intermediate salt formed:

$$R\text{–}COOH + (CH_3)_4N^+OH^- \rightarrow R\text{–}COO^-N^+(CH_3)_4 + H_2O \quad \text{Equation 4.2}$$

In the presence of water, therefore, the reaction will proceed more slowly.[1] For alkylation to proceed further, the intermediate salt must remain in solution. Methanol (MeOH) has been added to reaction mixtures to improve the solubility of the intermediate salt[10] although alkylation has also been reported to occur in MeOH-free reaction mixtures.[1] In the experiments of this study, TMAH was prepared as a methanolic solution.

Following the addition of an alkyl iodide to the reaction mixture, the corresponding alkyl derivative is formed with tetramethylammonium iodide salt (TMAI) precipitating.

$$R\text{–}COO^-N^+(CH_3)_4 + R_1I \rightarrow R\text{–}COOR_1 + N(CH_3)_4I \downarrow \quad \text{Equation 4.3}$$

where $R_1 = n\text{-}C_nH_{2n-1}$ representing any alkyl group

The alkyl iodide must be added to the reaction mixture only after the addition of TMAH. If not, the hydroxyl ions which catalyse the deprotonation of the acidic drug will react immediately with the carbon atom of the alkyl halide (nucleophillic substitution) and incomplete deprotonation of the acid may occur.[1,10]

The excess TMAH is neutralised as a result of the following reaction.

$$(CH_3)_4N^+OH^- + R_1I \rightarrow R_1OH + N(CH_3)_4I \downarrow \quad \text{Equation 4.4}$$

If the alkyl halide is sterically hindered by bulky groups, the reaction rate will be very low as the nucleophile must be able to approach the carbon atom for the reaction to proceed.[1] Similarly, a methyl halide is more reactive than higher *n*-alkyl halides as the electron density is less at the carbon atom under attack.[1] Alkylation reactions with alkyl halides are performed most commonly with iodides or bromides as the leaving group activity of $I^- > Br^- > Cl^- >> F^-$.[1] Iodine is displaced more easily than any of the other halides as it is the weakest base and is most stable as a free entity.[11]

Aprotic solvents provide the best medium for reaction as, in the presence of protic solvents, the nucleophile is stabilised by hydrogen bonding with the solvent molecules. In the literature, both dimethylsulphoxide and dimethylformamide (DMF) are used but DMF is preferred as it is more volatile.[5]
A diverse variety of drugs with acidic functional groups have been derivatised by quaternary ammonium hydroxide-catalysed alkylation reactions with alkyl

halides.[1,2] For example, butyl derivatives have been formed from a wide range of strongly to weakly acidic drugs (eg. barbiturates, phenytoin, ethosuximide, primidone, mefenamic and valproic acids, desmethyldiazepam, theophylline, paracetamol, ketoprofen, oxyphenbutazone, benzoylecgonine, probenecid and warfarin[1]).

Butyl derivatives are heavier compounds than methyl derivatives, with higher boiling points, as the molecular weight of the underivatised drug is increased by 56 or 112 amu (mono- and bis- derivatives respectively). Unlike diazomethane methylation, the occurrence of side-reactions in alkylation reactions with alkyl halides is rare,[2] and the reagents used have low toxicity.

4.2 Methods

Refer to "General Procedure" (Chapter 2). In particular, for HC marker solution refer to Section 2.2.5.5 and 2.2.5.6; for reference drug solutions refer to Section 2.2.5.14; for instrumentation refer to Section 2.4.1; and for SIM acquisition parameters refer to Section 2.4.3.1. Refer to Sections 2.4.2 and 3.2.1 for gas chromatographic parameters with the wider bore and narrow bore columns respectively. Qualitative and quantitative chromatographic data for drug derivatives was obtained using the method described in Section 2.6.1 unless otherwise specified.

4.2.1 Methylation

4.2.1.1 *Reagents*

methanol (HPLC grade); Mallinckrodt
redistilled diethyl ether; BDH Laboratory Supplies
Diazald (Aldrich Chemie, West Germany).
MethElute® (Pierce Chemical Co.); 0.2 M trimethylanilinium hydroxide in methanol
reference drug solutions; (Section 2.2.5.14)
HC marker solution; (Section 2.2.5.5)
ethereal diazomethane (freshly prepared)[12]

4.2.1.2 *Apparatus* (Section 2.3.2)

'quick-fit', non-ground connector glass-ware (Corning Incorporated) for diazomethane preparation.

4.2.1.3 *Derivatisation with Diazomethane*

All operations with diazomethane were carried out in a fume cupboard. Each working drug solution (100 µL) was transferred to a 2 mL glass reaction vial (Section 2.3.2) and evaporated to dryness under a stream of nitrogen at room temperature.

Ethereal diazomethane (100 µL) was added to the residue, the vial was sealed and allowed to react at room temperature for 30 min. On completion, it was noted that the solution was still yellow with unreacted diazomethane and the excess reagent was removed under a stream of nitrogen at room temperature.

Chloroform (100 µL) and the HC marker solution (100 µL) were added to the

residue, briefly vortexed (10 s) and the resulting solution transferred to inserts and autosampler vials for GC injection (Section 2.3.2).

Variations on the above procedure included the following:
1. the time of reaction - 0.5 and 2 h, and
2. the addition of MeOH as a catalyst - 50, 100 and 200 μL.

4.2.1.4 *Derivatisation with MethElute®*
In derivatisation experiments with MethElute® (0.2 M trimethylanilinium hydroxide in MeOH), 50 μL of the commercially prepared reagent was substituted for diazomethane (100 μL) in the reaction medium (Section 4.2.1.3). The reaction mixture was injected directly into the GC at an injector temperature of 270°C for on-column methylation of drug standards.

4.2.2 Butylation
Refer to Sections 2.2.3 and 2.2.5.4 for details of reagents and to Section 2.3.2 for apparatus employed; refer to Figure 2.4 for the derivatisation procedure. During development of the derivatisation procedure some parameters were varied as follows:-

1. the time of reaction - 15 min to 29 h,
2. the volume of TMAH added - from 20 to 150 μL,
3. the substitution of 0.1M tetrabutylammonium hydroxide (TBAH; 70 μL) for 0.1M TMAH (70 μL),
4. continuous agitation of the reaction mixture throughout the reaction time, and
5. heating (50°C) the reaction mixture throughout the reaction time.

The pH of the reaction mixture was monitored during experiments which investigated the application of heat throughout the reaction time. A drug free reaction mixture was prepared and, at the given time intervals (Section 4.3.2.1*v*), 50 μL removed and added to 3 mL of water. The pH of the aqueous solution was measured by a standard laboratory pH meter (pH electrode 1-14) and recorded.

4.3 Results

4.3.1 Methylation
4.3.1.1 *Diazomethane*
A selection of drugs, with a variety of functional groups and pKa values, were tested in preliminary alkylation experiments with diazomethane, and the results summarised in Table 4.1. Structurally-related drugs are grouped under headings which reflect a common chemical structure (Figure 3.1), and structurally-unrelated drugs are grouped where they possessed a common reactive functional group. The drug groupings used in this and other tables in the chapter are discussed in Section 3.3.1. Unlike the 'non-polar' nitrogen heterocyclic drugs shown in Tables 3.1-3.4, the drugs investigated during methylation have reactive secondary amine groups (Figure 3.2).

Carboxylic acids produced a single GC peak. The EI full-scan mass spectrum obtained from each GC peak enabled it to be identified as a methyl derivative (mono- or bis-). Where two sites were available for methylation (eg. enalaprilat), the bis- derivative was produced with a negligible yield of the mono-methyl derivative. The pKa values for these drugs ranged between 3.0 and 4.8.

Data from Table 4.1 show that drugs with weakly acidic functional groups (pKa ≥ 6), either formed no derivative or were incompletely derivatised. This was indicated by either the absence of any GC peak, or the presence of a GC peak identified as the underivatised drug. For example, diuretic agents (each possessing a sulphonamide grouping) and amides produced no methyl derivatives. Benzodiazepines were chromatographed predominantly as the underivatised drug although each produced GC peaks of negligible peak area identified as a methyl derivative. Allopurinol, captopril, methylphenobarbitone and phenytoin each formed methyl derivatives with significant peak areas. Their derivatisation was incomplete, however, as indicated by the presence of a small GC peak identified as the underivatised drug (except allopurinol). In the case of allopurinol which possesses two functional groups potentially susceptible to methylation, and for which the underivatised drug does not chromatograph (Table 3.1), incomplete derivatisation was indicated by the absence of the bis- derivative and chromatography of a single GC peak identified as the mono-methyl derivative.

Anti-diabetic agents which possess a sulphonylurea grouping (pKa 5 and 5.3) either produced no derivative (glibenclamide), or multiple peaks which were non-reproducibly formed (chlorpropamide, tolbutamide). Methyl derivatives of both chlorpropamide and tolbutamide were chromatographed together with underivatised and derivatised GC artefacts. The artefacts were identified as (4-chloro)- and (4-methyl)-benzenesulphonamide respectively (Section 5.3.2.3 and Table 5.6). Complicated peak profiles were also obtained for captopril and oxyphenbutazone which each produced three GC peaks of significant peak area identified as isomers of a bis- derivative.

Data shown in Table 4.1 indicated that derivatisation with diazomethane was most appropriate for drugs methylated at their carboxylic acid group. Despite relatively low pKa values for drugs with other functional groups, methylation either did not proceed, or did not proceed to completion as indicated by the presence of the underivatised drug, or mixtures of both mono- and bis- derivatives.

The effect of varied reaction conditions on incompletely derivatised drugs (nordiazepam, methylphenobarbitone, tolbutamide, oxazepam, bromazepam, sulindac) and a completely derivatised drug (ibuprofen) was investigated. Methanol (50, 100, 200 μL) was added to the reaction medium to catalyse methylation. However, increasing the volume of MeOH to 200 μL did not significantly increase the derivative yield for any drug. Similarly, increasing the time of reaction from 30 min to 2 h did not increase derivative yield for any drug.

4.3.1.2 *MethElute®*

The same selection of drugs investigated in experiments with diazomethane

(Table 4.1), were tested with a potent on-column methylating reagent - MethElute®. The commercially prepared reagent is a 0.2 M trimethylanilinium hydroxide in MeOH solution. When heated (hot GC injection at 270°C) with drugs possessing reactive amino, hydroxyl and carboxy functions, methylation occurs at these sites as illustrated by Figure 4.1.

MethElute® formed the same methyl drug derivatives as diazomethane, and with similar peak areas. For many drugs, however, derivatisation with MethElute® produced a host of additional peaks with EI full-scan mass spectra which were difficult to relate to either the underivatised drug, or its possible methyl derivatives. For example, ibuprofen (a carboxylic acid readily derivatised with diazomethane), produced two GC peaks. The EI full-scan mass spectrum and RI of one peak identified the mono-methyl derivative as formed with diazomethane. However, the second peak was found to have an identical EI full-scan mass spectrum but different RI despite the chemical structure of ibuprofen which indicates only a single derivatisation site. This could not be explained.

4.3.2 Butylation

The use of alkyl halides as alternative alkylating agents which successfully derivatise benzodiazepines is cited in the literature (Section 4.1). Preliminary experiments with *n*-butyl iodide indicated that benzodiazepines (nordiazepam, bromazepam, nitrazepam and clonazepam) which had been difficult to derivatise with either diazomethane or MethElute®, predominantly formed one mono-butyl derivative, with a negligible contribution from the corresponding isomer. Note that the benzodiazepine structure allows the possibility that tautomeric structures form (Figure 3.1). It was postulated that the reaction of drugs with *n*-butyl iodide may produce butyl derivatives of other weakly acidic drugs (pKa > 6), and acidic drugs (pKa 4.5-6) with functional groups other than carboxylic acids, which had been unsuccessfully derivatised with diazomethane.

Further investigation revealed, however, that some drugs which had readily formed methyl derivatives with diazomethane formed either no butyl derivatives (phenytoin, oxyphenbutazone), or multiple derivatives (eg. salicylic acid, allopurinol, diflunisal). Consequently, reaction and chromatographic conditions were explored to optimise the derivatisation process and chromatographic response for the majority of drugs (Sections 4.3.2.1–4.3.2.6).

4.3.2.1 *Optimising Reaction Conditions*

i. Time of Reaction

The effect of different reaction times (15 min to 29 h at room temperature; 100 µL TMAH; 100 µL *n*-butyl chloride) on the formation of butyl derivatives from selected drugs was followed by GC (Table 4.2). The GC peak reflected the formation (GC peak present or absent) and yield (increased or decreased GC peak height) of a butyl derivative. Drugs with strong acid functions (eg. ibuprofen, sulindac) reacted rapidly to produce a derivative after 15 min, although sulindac required an additional 30 min to form the maximum yield of its derivative (maximum peak height). Methylphenobarbitone, with weakly acidic properties, showed a similar pattern of derivative formation to that of sulindac with its

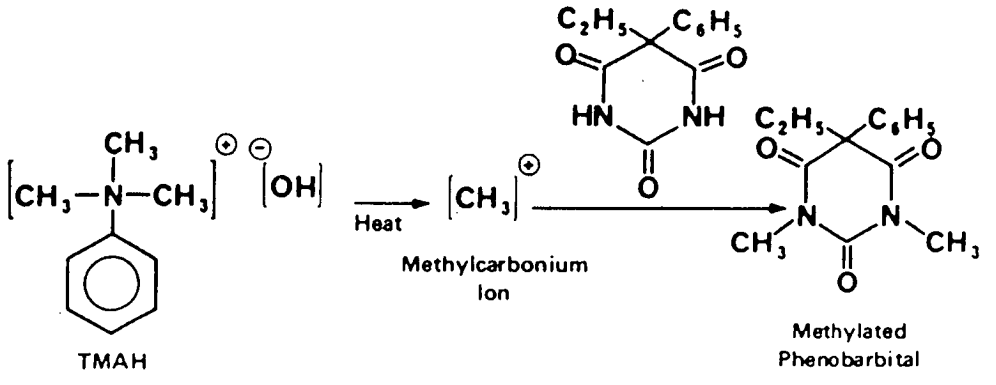


Figure 4.1: The mechanism of reaction of trimethylanilinium hydroxide (TMAH) with phenobarbitone to form a methylated derivative.

Figure was taken from educative material accompanying the on-column methylating reagent as supplied by PIERCE Illinios USA.

maximum peak height achieved at 45 min.

Data in Table 4.2 indicate that derivatives of the benzodiazepines (bromazepam, clonazepam, desmethyldiazepam oxazepam) with pKa ≥ 10.5 were first apparent following a reaction time of 45 min. Desmethyldiazepam and oxazepam each produced two peaks of approximately equal height identified as mono-butyl isomers. Further reaction time (2 h) resulted in an increase in the peak height of one of these isomers and a reduction in the other such that a single mono-butyl derivative predominated. In contrast, bromazepam and clonazepam each formed two mono-butyl derivatives after a reaction time of 45 min, one of which predominated. The peak heights of these derivatives did not change with additional time. Therefore, despite similarities in both structure and pKa, the formation of a single major butyl derivative appeared to have proceeded at different rates for individual benzodiazepines.

Following a reaction time greater than 2 h, there was no significant increase or decrease in the peak heights of butyl derivative/s for any drug tested.

ii. Volume of Catalyst

A selection of 26 drugs which possessed a variety of functional groups and pKa values, was tested in experiments which investigated the effect on derivative formation of different amounts of TMAH (20, 50, 70, 100 and 150 μL) in the reaction medium (100 μL *n*-butyl chloride; 2 h reaction time at room temperature;Table 4.3 - 'TMAH'). Any change in derivative formation for a given drug was reflected by a change in the corresponding GC peak/s. It was concluded that the maximum yield of a derivative had been achieved when the peak height did not change as the volume of TMAH increased.

Data in Table 4.3 indicate that, of the drugs tested, the carboxylic acids were the most susceptible to butylation as a GC peak was detected for each of them at the lowest volume of TMAH added to the reaction medium. More than one derivative was apparent for five of these drugs (benzoylecgonine, captopril, diflunisal, frusemide, sulindac).

When a greater volume of catalyst was added to the reaction mixture, the carboxylic acid derivative that was produced with only 20 μL TMAH either:-

1. decreased in peak height, or disappeared (captopril, frusemide, salicylic acid, sulindac), or
2. increased in peak height (benzoylecgonine, captopril, enalaprilat, ketoprofen, naproxen, sulindac, valproic acid), or
3. was unchanged (diclofenac, diflunisal, mefenamic acid, probenecid).

The GC peak heights of butylated carboxylic acids which had increased with an increase in TMAH volume, reached their maximum with 50 μL of TMAH and their yield did not alter when reacted with larger volumes.

For the majority of drugs with carboxylic acid groups, the addition of larger volumes of TMAH (50 to 150 μL) simplified their GC peak profiles from that

observed with the minimum volume of TMAH (20 μL). Under reaction conditions of ≥ 50 μL TMAH, a single major butyl derivative was produced and additional derivatives, if present, were of negligible peak height. The peak profiles of diflunisal, frusemide and salicylic acid, however, were further complicated by the addition of ≥ 70 μL TMAH, because new derivatives of negligible yield were formed.

Data in Table 4.3 show the effect on the GC peak height of derivatives formed from drugs with weaker acidic functions which had been reacted with different volumes of TMAH. Unlike the carboxylic acids, few of these drugs produced a derivative with the minimum volume of TMAH tested. For example, allopurinol produced mono-butyl derivatives with 20 μL TMAH which were replaced by bis-derivatives at larger volumes (50 to 150 μL). Similarly, although the mono-butyl derivative of tolbutamide (less its terminal propyl group as determined by the EI full scan mass spectrum) was detected following reaction with 20 μL TMAH, a significant GC peak was also identified as the underivatised GC artefact of tolbutamide which indicated incomplete derivatisation. Clearly, reaction with 20 μL TMAH was not sufficient to effect the butylation of weakly acidic drugs. Consequently, the formation of butyl derivatives from other weakly acidic drugs (eg desmethyldiazepam, methylphenobarbitone, phenytoin and primidone) was not tested at volumes of TMAH less than 70 μL (Table 4.3 - Note '3').

Warfarin and methylphenobarbitone were the only weakly acidic drugs tested which formed single butyl derivatives as might be predicted from their structures. All other drugs investigated formed two or more butyl related products. For the majority of drugs tested, 70 μL TMAH was sufficient to produce GC peak profiles with a single significant butyl derivative of maximum peak height with negligible peak heights for additional derivatives. Often, the major derivative was apparent with 50 μL TMAH but maximum peak height was achieved with a 70 μL volume. Allopurinol, however, produced two significant bis- derivatives with all volumes of TMAH tested (50 to 150 μL).

As with the carboxylic acids, volumes of catalyst greater than 70 μL (100 and 150 μL) were not detrimental to butyl derivatives formed from weakly acidic drugs regardless of the functional group derivatised, and no change in peak height was observed except for bromazepam, clonazepam, tolbutamide. For both bromazepam and clonazepam the decrease in peak height did not occur until 150 μL TMAH was used and, for tolbutamide, it was the mono-butyl derivative which decreased, simplifying the peak profile for that drug.

The butylation of the benzodiazepines was generally consistent in the formation of mono-butyl isomers, one of which was of negligible peak height, and the other with a peak height which reached its maximum with 70 μL TMAH. Oxazepam and lorazepam departed from this pattern with the appearance of a GC artefact of the underivatised drug thought to be formed in the injector[13] and, in the case of oxazepam, the occurrence of both mono- and bis- derivatives.

Data shown in Table 4.3 indicate that for the majority of strongly and weakly

acidic drugs tested, 70 µL TMAH was adequate to ensure the formation of a single significant butyl derivative, with a negligible contribution to the peak profile of additionally formed derivatives.

iii. Substitution of TBAH for TMAH

Butylation with an alkyl halide requires a tetra-alkyl ammonium hydroxide as a catalyst (Section 4.1.3). The effect on the GC peak heights of butyl derivatives produced by the exchange of 70 µL TMAH for 70 µL tetra-butyl ammonium hydroxide (TBAH) was investigated (Table 4.3 - 'TBAH'). For all 26 drugs investigated, the effect of catalyst substitution on the GC peak heights of the major butyl derivatives was minimal regardless of pKa or the functional group derivatised.

Where there were two or more derivatives formed from a drug, the peak heights of minor derivatives (negligible peak height) showed some variation (increase or decrease). For example, tolbutamide, primidone and oxyphenbutazone showed increases in the peak heights of minor derivatives, further complicating the peak profiles for those drugs. In contrast, the GC peak profiles of lorazepam, salicylic acid, captopril, diflunisal, phenytoin and nitrazepam were simplified following decreases in the peak heights of minor derivatives.

It was observed that tetrabutylammonium iodide (TBAI; salt by-product of derivatisation; Equation 4.3) was soluble in the GC injection solvent. Consequently, TBAI would be deposited in the injector of the GC with each sample injected. In contrast, TMAI was insoluble and remained behind when the GC extract was transferred from the reaction vial to the autosampler vial prior to GC analyses.

In all subsequent analyses, 70 µL TMAH was used to catalyse the butylation of drugs with *n*-butyl iodide.

iv. Agitation

It was observed during the investigation of reaction times that the precipitation of TMAI salt was slow (up to 1h), and yet, if the reaction vial was briefly vortexed, precipitation of the salt followed. The literature states that progression of derivatisation was aided by agitation[1] and so continuous agitation was also investigated. It was found that continuous agitation throughout the reaction time had no effect on the peak heights of derivatives formed for any of the 26 tested drugs regardless of their pKa or the functional group.

v. Heat

Figure 4.2 shows the changing pH of a drug free reaction mixture (Section 4.2.2) following reaction at room temperature and the application of heat (50°C; 15 min). The pH of the reaction mixture decreases as excess TMAH is removed by the formation of TMAI salt (Equation 4.3 and 4.4).

At the beginning of the reaction at room temperature, the mixture was strongly alkaline (pH 10.45 ± 0.05; n = 3) and the pH remained high (> 9.4) for up to

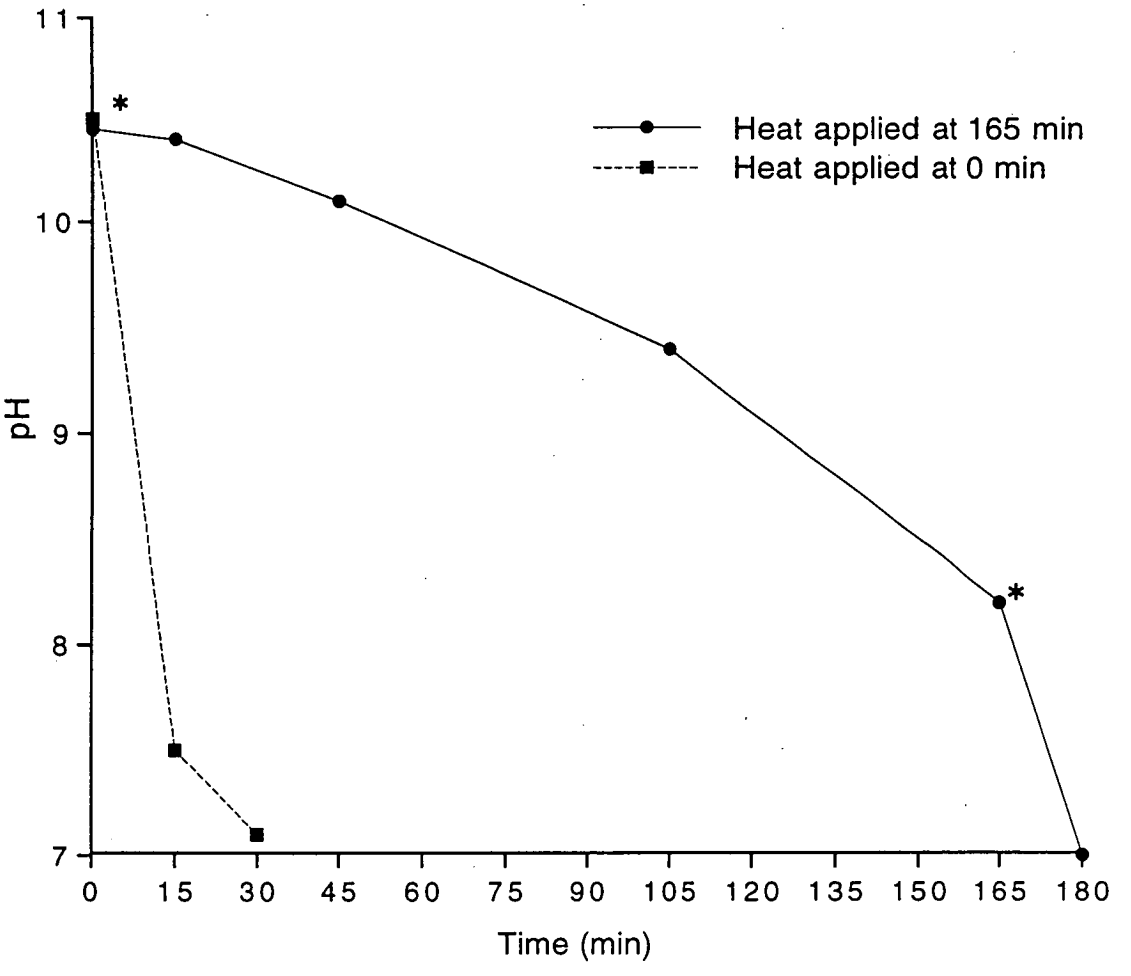


Figure 4.3: The change in pH (± 0.05; $n = 3$) of the drug-free reaction mixture with time and following the application of heat* (50°C for 15 min).

105 min. Following reaction for a further 60 min at room temperature, the pH decreased only marginally from that at 105 min, to pH 8.2. Consequently, at the completion of the normal 2 h reaction time (120 min), the mixture injected into the GC would be approximately pH 9. With the application of heat at 165 min (50°C; 15 min), the pH of the reaction mixture fell to 7.0.

To a second drug-free reaction mixture (pH 10.5), heat (50°C; 15 min) was applied immediately and the pH of the reaction mixture dropped to 7.5 (Figure 4.2). With a further 15 min at room temperature, the pH decreased to 7.1. Clearly, the removal of excess TMAH from the reaction mixture was aided by the application of heat.

Table 4.3 shows the effect of a final heating step (50°C for 15 min following reaction at room temperature for 2 h) on the peak heights of butyl derivatives for the 26 test drugs. For the majority of strongly acidic drugs the effect was minimal with no change recorded in the peak heights of their derivatives. The following exceptions were observed.

1. Diflunisal (peak height of the bis- derivative was reduced) and salicylic acid (no methyl- or methyl-butyl derivatives were formed). These changes were beneficial and resulted in simplified peak profiles.
2. Sulindac (peak height of an unidentified related product increased).

Of the drugs tested with weakly acidic functional groups, the phenols, warfarin, tolbutamide, methylphenobarbitone, and some benzodiazepines (bromazepam, desmethyldiazepam, nitrazepam) showed no significant change in the peak heights of corresponding butyl derivatives following the application of heat. The following exceptions were observed.

1. Lorazepam and phenytoin (peak height of the most significant derivative was so reduced by heat, that another derivative predominated).
2. Clonazepam (an increase in the peak height of the most significant butyl derivative).
3. Oxazepam (the peak profile was simplified by the reduction in peak height of negligible derivatives).
4. Primidone (maximum peak height of the bis- derivative occurred at 70 rather that 100 μL TMAH).

Consequently, the effect on derivative peak height with the application of heat to the reaction mixture for these drugs was either beneficial (clonazepam, oxazepam, primidone) or of little significance (lorazepam, phenytoin).

The effect on derivative peak height with prolonged heating was investigated (the reaction mixture was heated at 50°C for 2 h). The effect on derivative peak heights following prolonged heating was minimal compared with brief heating (15 min) for the majority of drugs tested regardless of their functional group or pKa. Primidone and diflunisal, however, did show a reduction in the peak heights of derivatives of negligible size which appeared only when volumes of TMAH > 70 μL were used.

Consequently, to avoid injecting a strongly alkaline solution into the GC and, as prolonged heating throughout the reaction time was not advantageous, reaction at room temperature followed by brief heating (50°C; 15 min) was used in all subsequent butylation experiments.

Having established the optimal reaction conditions which would produce butyl derivatives for the majority of test drugs, other drugs selected for their potential to form butyl derivatives were investigated on the narrow bore column (Table 4.4).

As might be expected from data shown in Table 4.3, carboxylic acids, the barbiturates and ethosuximide (very similar to phenytoin) formed single butyl derivatives, and the peak heights of additional peaks (where present) were negligible. The butyl derivative of an indomethacin artefact was formed. As no butyl derivative of the drug was apparent, it is postulated that the drug was completely converted to the artefact during reaction, and was subsequently butylated. Methyprylone (pKa 12.0) formed no butyl derivative.

Those drugs listed in Table 4.4 for which no butyl derivative was formed were also those for which derivatisation with diazomethane was unsuccessful (Table 4.1). Metoclopramide and procainamide where both chromatographed as underivatised drugs and no butyl derivatives were formed. Glibenclamide and drugs with sulphonamide groupings (diuretic agents) formed either no derivative, or multiple unidentified related products of negligible peak heights. It was concluded, therefore, that these drugs were not susceptible to alkylation by either *n*-butyl iodide or diazomethane under the present reaction conditions.

As might be expected from data shown in Table 4.3, the benzodiazepines shown in Table 4.4 also formed a single major butyl derivative with a negligible peak chromatographed for the alternate isomer.

The butylation and chromatography of chlorpropamide was the same as that of tolbutamide - GC peaks corresponding to a derivatised and underivatised artefact (Table 5.6) were present together with the mono-butyl derivative of the drug (less the terminal ethyl group as determined by the EI full-scan mass spectrum).

4.3.2.2. *Reproducibility of Derivative Formation*
Data in Table 4.5 indicate the reproducibility of derivatisation of major butyl derivatives formed from some of the drugs investigated in Tables 4.3 and 4.4.

Working drug solutions were prepared and three samples dispensed into reaction vials. Two of the samples were derivatised and analysed within the same day and the third sample was derivatised and analysed the following day (Section 2.6.1). The 'within day' data show the percentage difference in the relative peak areas between replicates (R1 and R2), and 'between day' data indicates the percentage by which the second day sample (S) differed from R1. Although the reproducibility tests compare only single samples within and between days, the data does give an indication of which derivatives were formed very reproducibly and which were not.

In general, 'within day' data indicated that derivatisation at the carboxylic acid group was reproducible, with variations in peak area of 25%. Those derivatives for which the 'within day' variation was greatest (captopril, diflunisal, salicylic acid, sulindac) were also those derivatives for which additional derivatisation products were formed (related products and/or mono- and bis- derivative mixtures; Tables 4.3 and 4.4). Of these, the 'within day' data indicate the poor reproducibility of derivative formation for mono-butyl derivatives of diflunisal and sulindac. Similarly, the 'between day' variation in derivative formation for carboxylic acids was also greatest for diflunisal and sulindac. Note that mono-butyl diflunisal was the major derivative formed for this drug (Table 4.3).

Of the less acidic (pKa > 6) drugs tested, all the barbiturates were reproducibly butylated both within, and between days. The 'within day' variation was marginal for these drugs (from 0 to 8%) and the 'between day' variation only slightly greater (from 6 to 22 %). Butyl derivatives of phenols appeared to be reproducibly formed within a day, but showed greater variation between days, particularly allopurinol and paracetamol. The majority of butyl benzodiazepines were formed reproducibly. Derivatives of both drugs and their metabolites (except clonazepam and lorazepam) showed less than 23% variation in peak areas within a day, and slightly greater variation between days (up to 35%). Clonazepam, and lorazepam were the least reproducibly derivatised within a day. The between day formation of their derivatives, however, was similar to all other benzodiazepines tested. Anti-diabetic agents derivatised at their sulphonylurea grouping (approximately pKa 5) showed poor reproducibility both within and between days. Only the mono-butyl derivative of the GC artefact of tolbutamide was reproducibly formed.

Despite general similarities in the reproducibility of formation of butyl derivatives for drugs within each group (eg. carboxylic acid and barbiturate derivatives were formed reproducibly; sulphonylurea derivatives were not), the reproducibility of formation of a number of individual drug derivatives within a group differed. For example, the range of variation between replicates within a group was quite broad with the 'within day' variation for carboxylic acids and benzodiazepines ranging from 0% to 26%, and 0% to 23%, respectively. In addition, some drugs with similar functional groups generated reproducibility data quite different from others of the same function (eg. diflunisal and sulindac, paracetamol, clonazepam, chlorpropamide). Clearly, the structure and chemistry of individual drugs was important in determining the reproducibility of derivative formation at a particular functional group.

4.3.2.3 *Chromatography of Butyl Derivatives on Narrow and Wider Bore Columns.*

i. Differences in Derivatives Detected

Table 4.6 shows chromatographic data for butyl drug derivatives on both the narrow and wider bore columns (Section 4.2). A number of additional butyl drug derivatives not previously investigated are listed. These derivatives were included for one of the following reasons:-

1. the derivative was formed from a target drug which was selected through the process described in Section 1.2. (eg. tiaprofenic acid);
2. the derivative was discovered unexpectedly during the investigation of drug extraction procedures (Chapter 7) and its chromatographic characteristics are included for completeness (eg tricyclic anti-depressant agents);
3. the derivatives was detected on the wider bore column but was absent on the narrow bore column (eg. tri-butyl frusemide).

For some drugs, the peak profiles of derivatisation products chromatographed on the wider bore column differed from those observed on the narrow bore column. On the wider bore column it was noted that:-

1. a late eluting peak identified (EI full-scan mass spectrum) as tri-butyl frusemide was chromatographed;
2. bis- derivatives of several drugs were identified (chlorpropamide and tolbutamide GC artefacts, desmethylclobazam, ketoprofen, phenytoin and salicylic acid);
3. an alternate lorazepam derivative was chromatographed, and the derivative that was detected on the narrow bore column was chromatographed as multiple unresolved peaks;
4. multiple unresolved peaks in addition to the bis- derivative of allopurinol were chromatographed;
5. the lorazepam GC artefact detected on the narrow bore column was absent;
6. a bis- derivative of oxazepam was detected which replaced the mono-butyl derivative detected on the narrow bore column; the GC artefact, however, was detected on both columns.

In cases where the GC peak profile for derivatives of a drug differed between the two columns, it was found that one of the following circumstances applied.

1. The major derivative chromatographed on the wider bore column was the same major derivative detected on the narrow bore column. Although the additional derivative detected on the wider bore column was of negligible peak height, a more complicated GC peak profile resulted for that drug (eg. allopurinol, desmethylclobazam, ketoprofen, phenytoin).
2. The major derivative chromatographed on the wider bore column was not the same major derivative detected on the narrow bore column. For both chlorpropamide and tolbutamide, the bis- derivative of the GC artefact predominated; for oxazepam, primidone, salicylic acid and sulindac, the bis-derivative predominated; for frusemide, the tri-butyl derivative predominated, for lorazepam, the late-eluting isomer predominated.

Table 4.7 compares the calculated retention indices determined on both columns for 41 butyl drug derivatives. Derivative RI values cover a wide range (1300 to 3200). In all cases the calculated RI for a specific derivative was greater on the narrow bore column than on the wider bore column. Table 4.7 shows the difference between the RI values determined on each column (N - W). As was observed in Table A.2 for underivatised drugs, the difference in RI between the two columns appeared to increase with increasing carbon number but the increase was neither consistent nor predictable for derivatives with similar RI values.

ii. Linearity

Data in Table 4.8 show quantitative and qualitative chromatographic data for selected butyl derivatives chromatographed on both the narrow and wider bore columns. The majority of the quantitative data relates to narrow bore column chromatography, although some data determined on the wider bore column is included where a derivative was either:-

1. not investigated on the narrow bore column (eg. nortriptyline, tiaprofenic acid), or
2. quantitatively differed from data determined on the narrow bore column (eg. benzoylecgonine).

Qualitative data determined on the narrow bore column is given for most derivatives with regard to the shape of the peak (A), carry over from the injector to a following sample (B), and the amount injected (when known) which caused column overload [x]. Similar data is given for derivatives chromatographed on the wider bore column where it differed from that determined on the narrow bore column.

As described in Section 2.6.1, each data point employed in determining linear regression equations was a peak area ratio. Table 4.8 lists both the target *m/z* ion monitored and the reference HC marker from which the peak area ratios were calculated for individual butyl derivatives. Linearity data over a defined concentration range is shown. The concentration range is defined by the 'highest' and 'lowest' drug amounts. The 'lowest' amount injected refers to the smallest drug amount that could be integrated. One hundred- to 1000-fold differences in amounts injected were chromatographed for the majority of butyl derivatives.

The linear regression equation shown for each derivative described the line of best fit for the $\log_{10}$ of the data points - ie. $\log y$ (y = peak area ratio) against $\log x$ (x = drug amount injected). As for underivatised drug data (Tables 3.2 and 3.4) the log transformed data points for each derivative were fit by a linear regression curve over a concentration range between, and including, the 'lowest' and 'highest' amounts injected as listed in Table 4.8. The linear regression equation for log-transformed data points and its relationship ($y = 10^{C}.x^{M}$) to untransformed data points is discussed in Section 3.3.1.

Data in Table 4.8 show that the majority of butyl derivatives tested were chromatographed on both columns with good peak shape, regardless of the functional group derivatised. The GC peaks chromatographed were, at best, Gaussian in shape, and at worst, slightly tailing. Only the lorazepam artefact and the mono-butyl derivative of primidone, had moderately tailing peak shapes on the narrow bore column. Following chromatography on the wider bore column, the peak shapes of some derivatives were marginally improved from those chromatographed on the narrow bore column (eg. the barbiturates).

Carry-over from a previous injection was minimal for all derivatives. Column overload, as indicated by peak shape and the plateauing of the detector response, did occur on the narrow bore column for butyl derivatives of ibuprofen, indomethacin, salicylic acid and tolbutamide. Due to differences in column capacity between the narrow and wider bore columns (Appendix B.3.2.3), column overload occurred for several derivatives chromatographed at their highest

amounts on the wider bore column (eg several barbiturates and carboxylic acids).

A power equation ($y = 10^C.x^M$) describes the linear data points in terms of the $\log_{10}$ transformed data ($Y = MX + C$) and this equation is linear when $M = 1.0$ (Section 3.3.1). Table 4.8 gives the 10^M factor which is a good indicator of derivative linearity over the concentration range tested (Section 3.3.1).

Linearity data (10^M) in Table 4.8 indicate the following.

1. Data points for 31% of butyl derivatives investigated were best fitted (r^2 from 0.995 to 1.000) by an approximately linear equation (10^M from 8.3 to 12.9):- all the barbiturates, chlorpropamide artefact (bis), clobazam, diclofenac, diflunisal, ethosuximide, ibuprofen, nortriptyline, salicylic acid (bis), theophylline, tiaprofenic acid and tolbutamide. Warfarin data points were also described by a linear equation, however, the data points were not as well fitted (r^2 0.932).
2. Data points for 30% of butyl derivatives were best described by a gently sloping curve where the increase in detector response was greater for larger drug amounts injected (10^M from 13 to 20):- desipramine, desmethyldiazepam, diflunisal (bis-), indomethacin, ketoprofen, lorazepam, metoclopramide, nitrazepam, oxazepam, oxyphenbutazone, phenytoin, probenecid, salicylic acid, temazepam, tolbutamide.
3. Data points for three butyl derivatives (5%) were best described by with a gently sloping curve where the increase in detector response was greater for smaller drug amounts injected (10^M from 5.1 to 6.8):- lorazepam, sulindac, valproic acid.
4. No linearity data on either column was obtained for three butyl derivatives (5%) as only two data points were obtained over the concentration range tested.
5. Data points for 27% of the butyl derivatives chromatographed on the narrow bore column had $10^M > 20$, except 7-amino clonazepam and enalaprilat, where $10^M = 1.8$ and 2.0 respectively. Many of these were benzodiazepine and their metabolites plus captopril, enalaprilat, benzoylecgonine, morphine, protriptyline and quinine. These drugs were also injected at relatively high concentration compared with other drugs with the lowest amount detected usually in the nanogram range.
6. The linearity of some butyl derivatives (benzoylecgonine, some benzodiazepine metabolites and valproic acid) was improved following their chromatography on the wider bore column from that determined on the narrow bore column. In all instances, the 10^M factor indicated an improvement in linearity over the concentration range tested. For the majority of butyl derivatives, however, the 10^M factors were similar between columns.

For butyl derivatives with approximately linear characteristics, a 1000-fold increase in concentration on the narrow bore column and a 100-fold increase on the wider bore column was investigated. For the majority of other derivatives tested, data points were best fitted by a curve ($M \neq 1$). Of these, two-thirds of the derivatives had data points which covered a 100- to 1000-fold increase in concentration, while for a third, the concentration increase was only 10-fold.

iii. Detection Limit

Data in Table 4.9 shows the lowest amount injected that produced a peak which could be integrated ('detected'), and the next lowest amount injected that did not produce a peak which could be integrated ('not detected') for 52 butyl derivatives chromatographed on both narrow and wider bore columns. Forty drugs are listed for which data on both columns was obtained. The limit of detection for a given drug fell between the amounts listed in the 'detected' and 'not detected' columns and is an indicator of differences in the chromatographic response of the derivative on each column.

Data in Table 4.8 indicated that 20% of derivatives chromatographed on the wider bore column showed a decreased limit of detection (increased chromatographic response) from that achieved on the narrow bore column and, for three, the decrease was ten-fold (desalkylflurazepam, desmethylflunitrazepam and frusemide). The majority (65%) of butyl derivatives achieved similar limits of detection when chromatographed on either the narrow or wider bore column (Table 4.9). Data indicated that 15% of derivatives chromatographed on the wider bore column showed an increased limit of detection (a poorer chromatographic response) from that achieved on the narrow bore column (Table 4.9). These data differ from similar data obtained for underivatised drugs on the two columns (Table B.12). Data in Table B.12 indicated that 47% of underivatised drugs had an increased chromatographic response (decreased detection limit) following their chromatography on the wider bore column while, for 46% of drugs, instrument sensitivity was similar.

Data in Table 4.9 indicate that 85% of the butyl derivatives chromatographed on the wider bore column had chromatographic responses similar to, or greater than, those obtained on the narrow bore column.

4.4 Discussion

The suitability of a variety of drugs to undergo alkylation reactions, and the chromatography of many alkyl drug derivatives on the narrow and wider bore columns, was explored. Alkylation was possible where the drug possessed a polar functional group with an acidic proton which could be replaced by a non-polar alkyl group. Consequently, the drugs considered for derivatisation through alkylation were those with any acidic functional grouping even though the relative acidity of the drug was weak (eg. R-COOH, R-SO_2OH; R-OH, R-SH, R-NH_2, R_2NH, $RCONH_2$, RSO_2NH_2).[5]

It could be anticipated that the lower the pKa of a drug, the greater would be the potential for that drug to be derivatised by an alkylation reaction. Tables 4.1 and 4.4 gave pKa values for many of the drugs investigated, however, during the development of suitable derivatisation conditions, it was soon apparent that pKa was not the best indicator of the potential of individual drugs to undergo derivatisation.

4.4.1 Methylation

Table 4.1 listed the methyl derivatives formed from selected drugs with different functional groups and pKa values following their reaction with diazomethane. Strongly acidic drugs were methylated at their carboxylic acid groups and produced GC peaks identified as mono- derivatives. In a few cases, a bis-derivative was also identified indicating that a second acidic proton was replaced at another susceptible functional group (in addition to the carboxylic acid group). Clearly, the acidic proton was readily dissociated in the presence of diazomethane (Equation 4.1), as predicted by their relatively low pKa. Many of the weakly acidic drugs (pKa > 6) were either incompletely methylated, or formed no derivative.

Consequently, MeOH was introduced to catalyse the reaction. Methanol acts to both catalyse methylation by diazomethane and improve the solubility of the drug in the reaction mixture.[2] It was shown, however, that the inclusion of MeOH in the reaction medium produced no change in the GC peak heights of either methyl derivatives or underivatised drugs chromatographed (Section 4.3.1.1). Clearly, the rate limiting factor in the methylation of these drugs by diazomethane was related to parameters other than those that could be improved/catalysed by MeOH. Similarly, when reaction time was increased four-fold (from 0.5 to 2 h), there was no increase in the peak heights of methyl derivatives or corresponding decrease in peak heights of underivatised drugs co-chromatographing with them.

Chemical reactions are driven by the formation of reaction products which are stable at lower energy states than the reaction precursors in the current environment of the reaction medium.[11] It is postulated, therefore, that drugs for which no methyl derivatives were detected by GC:-

1. were more stable as the underivatised drug than as methyl derivatives in reaction conditions created by the drug and diazomethane;
2. were difficult to deprotonate and required a catalyst other than MeOH to cause deprotonation;
3. were very slow to react with diazomethane under the present reaction conditions requiring either a reaction time greater than 2h, or the addition/adjustment of parameter/s not investigated to increase the reaction rate; or
4. produced methyl derivatives which were very susceptible to thermal decomposition and which entirely decomposed either in the hot injector or during chromatography.

Alkylation with MethElute® was briefly explored as it provided a very different reaction environment from that provided by diazomethane. It was found to be no more effective in the methylation of the test drugs, however, than was diazomethane. It is postulated that factors such as derivative stability and ease of deprotonation of the underivatised drug were prohibiting the formation and/or detection of methyl derivatives. Multiple additional peaks were detected in the chromatograms following reaction with MethElute®. The high injector temperature (270°C) required for successful methylation catalysed side-reactions within the derivatising mixture to produce a host of unidentified peaks. The mass

spectra of the end-products could not be easily related to the underivatised drugs which complicated the analysis of chromatograms.

Clearly, alkylation with either diazomethane or MethElute® was not appropriate for drugs other than those derivatised at a carboxylic acid group.

The routine methylation of drugs prior to GC analysis has one distinct disadvantage in that methylated derivatives could be confused with other underivatised drugs present in the blood specimen. For example, caffeine (methyl-theophylline) would be formed from theophylline. Similarly, some drugs (eg. diazepam, flurazepam, imipramine, amitriptyline, clobazam, methylphenobarbitone) are metabolised in the body to produce desmethyl compounds and, if methylated, these desmethyl-metabolites would be indistinguishable from the parent. Some of the desmethylated metabolites are manufactured as pharmaceutical products (desmethyldiazepam, desipramine, nortriptyline, phenobarbitone) and following their methylation, confusion would exist as to which drug had been originally administered.

Another disadvantage associated with the GC/MSD analysis of methyl derivatives is that derivatisation does little to alter the EI full-scan mass spectra from those obtained from underivatised drugs (eg. M^+ increased by only 14 or 28). The EI full-scan mass spectra of many of the low molecular weight drugs (eg. salicylic acid, valproic acid, ethosuximide) and their methyl derivatives contain relatively small *m/z* ions which may not be characteristic enough to distinguish them from *m/z* ions produced by the endogenous compounds in blood and plasma. Consequently, endogenous compounds may potentially interfere with the detection and identification of these derivatives in biological samples.

4.4.2 Butylation

4.4.2.1 *Introduction*

Tables 4.3, 4.4 and 4.6 listed the butyl derivatives formed from selected drugs with different functional groups and pKa values following their TMAH-catalysed butylation with *n*-butyl iodide. As with diazomethane methylation, strongly acidic drugs (pKa from 3.0 to 4.8) were butylated and produced single GC peaks identified as either mono- or bis- derivatives. In contrast with diazomethane, however, some weakly acidic drugs (eg. diuretic agents with sulphonamide groupings) also formed several butyl products related to the drug.

4.4.2.2 *The Reaction*

Acidic compounds can be alkylated when they are present in their anionic form provided that the nucleophilicity of the conjugate base is sufficient to displace the leaving group (iodide) from the saturated C atom of *n*-butyl iodide (Section 4.1.3). The properties of the nucleophile, leaving group, reaction solvent and substrate all contribute in governing the rate of S_N2 reactions.[11]

In the butylation of drugs listed in Tables 4.3 and 4.4, the leaving group, substrate and solvent were the same for every reaction while the structure of the nucleophile (drug) varied greatly. It is likely, then, that the formation (or not) of butyl

derivatives from the different drugs investigated was largely dependent on the properties of individual drug nucleophiles.

Apart from the overall structural diversity of the drug nucleophiles participating in butylation reactions, there was also some variation in the atom attacking the saturated C atom of *n*-butyl iodide (oxygen, nitrogen, sulphur). Oxygen was the attacking atom for drugs butylated at a carboxylic acid, hydroxyl or phenolic group. For barbiturates, sulphonylureas and sulphonamides, nitrogen was the attacking atom. In the butylation of one of the active sites of captopril, sulphur was the attacking atom. In addition, some drug nucleophiles contained more than one attacking atom (forming bis- derivatives) which further influenced the rate at which the alkylation proceeded.

The following general properties of a nucleophile affect the rate of S_N2 reactions.[11,14]

1. In comparing nucleophiles whose attacking atom is in the same row of the periodic table, the nucleophilicity is approximately in order of basicity particularly when the structures of the nucleophiles are similar. Consequently, decreasing basicity follows $R\text{-}NH_2 > RO^- > OH^- > R_2NH > ArO^-$. However this is not exact because the most effective nucleophile is the one whose attacking atom has valence electrons most available for coordination with the C atom (nucleophilicity) rather than H^+ (basicity).[14]
2. In comparing nucleophiles whose attacking atom is in the same column of the periodic table (eg oxygen and sulphur) then nucleophilicity is greatest for the larger atom in a protic solvent. The smaller the atom, the more concentrated is the negative charge, and the more tightly it is bound by hydrogen bonding with solvent molecules. Consequently any sulphur nucleophile is more powerful than its oxygen analog.
3. The freer the nucleophile from intermolecular bonds stabilising electron density at the nucleophilic centre (eg weak bonds associated with solute and solvent - 'solvation'), the greater the rate of reaction.

In addition to these general principles, however, steric influences often play a major part in determining the rate of reaction as, the larger the bulk of the nucleophile, the greater is the difficulty experienced in approaching and attacking the saturated carbon of *n*-butyl iodide.

As stated above, the greater the density of electrons at the nucleophilic centre, the greater its reactivity (nucleophilicity). The presence of electron attracting substituents on the nucleophile slow the reaction as they drain the electron density away from the nucleophilic centre[11,14] and this 'inductive' effect decreases with distance from the nucleophilic centre. Consequently, the inductive effect is most pronounced when the electron attracting substituent is *ortho* to the nucleophile on an aromatic ring or on the α and ß carbons in aliphatic compounds.

Similarly, resonance effects in aromatic systems increase or decrease electron density by either supplying electron density to the system (halogens, -OH, -OR, -OOCR,-SH, -SR, $-NH_2$, $-NR_2$, -NHCOR), or withdrawing it ($-NO_2$, -CN,

-CHO, -COR, -COOH, -COOR, -$CONH_2$, -SOOR, -CF_3).[11,14] These resonance effects are most apparent when the substituent is *para* to the nucleophilic centre otherwise a combination of both the inductive and resonance effects of the substituent affect nucleophilicity. Resonance effects may also be sterically hindered. Resonance effects are greatest when the substituent groups are in the same plane as the nucleophilic centre, however, when this is not the case, the electron withdrawing or supplying nature of the substituent group is decreased.[11,14]

The electron density at the nucleophilic centre can be stabilised not only by solvation but, where structure permits, by intramolecular hydrogen bonding.[10] For example, salicylic acid has a hydroxy group *ortho* to the -COOH group. Following loss of the proton, hydrogen bonding occurs to spread the negative charge across the hydrogen bridge rather than localising it to the carboxylate group[14] decreasing the reactivity of the nucleophile.

Consequently, the nucleophilicity of the conjugate base (deprotonated drug) is dependent on:-

1. the chemical nature of the attacking atom,
2. the unsaturated substituents of the attacking atom, and
3. the ability of these substituents to delocalise the negative charge remaining on the attacking atom after the proton is removed.

The attacking atom and its substituents, other functional groups carried by the nucleophile, its size and the space it occupies (bulk), the strength of inductive and resonance effects around the nucleophilic centre, solvation and intramolecular hydrogen bonding each affect the reactivity of an individual drug nucleophile to a degree which would be difficult to predict. Consequently, the comparison of structurally-unrelated drug nucleophiles participating in alkylation reactions would also be extremely difficult.

4.4.2.3 *Butyl Drug Derivatives*

Data in Table 4.2 showed the effect of different reaction times on the formation of selected butyl derivatives. As deprotonation of the drug in the presence of TMAH is instantaneous (Equation 4.2), the reaction time required to form butyl derivatives reflected the ease with which the nucleophile approached the saturated C atom of *n*-butyl iodide and displaced I^- (nucleophilicity). Therefore, the rapid formation of butyl derivatives of ibuprofen, sulindac and methylphenobarbitone indicated that these drugs produced nucleophiles with greater reactivity than the nucleophiles of the benzodiazepines (Table 4.2). The benzodiazepine nucleophiles displaced I^- to form butyl derivatives but the rate of the S_N2 reaction was slower than that of the other drug nucleophiles. In addition, data in Table 4.2 showed that the reaction rate of structurally-related nucleophiles (benzodiazepines) was similar but not the same, indicating that even subtle structural differences in the chemistry of these nucleophiles resulted in differences in reaction rate. These data tend to support the conclusion that many interacting factors govern the rates of S_N2 reactions (Section 4.4.2.2)[11]. Data in Table 4.2 also indicate that long reaction times for drugs which were butylated rapidly was

not detrimental to the resulting derivatives as no decrease in peak heights was observed. Consequently, the derivatives produced were stable in the conditions created by the reaction mixture and no additional side-reactions occurred.

In all butylation experiments, TMAH in MeOH (a protic solvent) was added to the reaction mixture. With different volumes added to the reaction mixture (from 20 to 150 μL), both the volume of protic solvent and the molar ratio of TMAH to *n*-butyl iodide changed. For the majority of test drugs, the greater the volume of TMAH added, the more strongly the equilibrium of the reaction was shifted in the direction of forming the nucleophile. At the same time, however, the greater the amount of TMAH added, the greater the volume of MeOH concurrently added to the reaction mixture with the potential to stabilise the electron density at the nucleophilic centre through hydrogen bonding (potentially decreasing nucleophilicity). Data in Table 4.3 showed that more than one derivative was formed for some drugs and the relative peak areas of the multiple derivatives changed with changing amounts of TMAH such that some completely disappeared while others were newly formed. Clearly, the formation of the nucleophile by deprotonation and its relative nucleophilicity in the reaction solvent governed the changes in peak height of the resulting derivatives.

The removal of excess TMAH from the reaction mixture was important as its presence would make the final solution for GC injection strongly alkaline and, consequently, its presence would be detrimental to both the injector and GC column. Hence the addition of the final heating step (Section 4.3.2.1*v*).

The substitution of TBAH for TMAH did not affect the butylation of the test drugs in any significant way. This result indicated that neither the deprotonation of the drug, the solubility of the intermediate salt, nor the reactivity of the nucleophile was significantly affected by the higher *n*-alkyl quaternary ammonium hydroxide. The formation or not of butyl derivatives was dependent on the corresponding nucleophile and its nucleophilicity as governed by individual drug chemistry. For some drugs, there was either an increase or decrease in the peak heights of minor products. The decreased peak heights of minor products was observed for six drugs and would be advantageous as simplified peak profiles would result for these drugs. The increase or decrease in peak height of a derivative which contributed negligibly to the total peak area of derivatives formed, however, would convey very little advantage or disadvantage.

Preliminary experiments indicated that for a range of drugs (allopurinol, valproic and salicylic acids, captopril, benzodiazepines) the peak heights of butyl derivatives formed were extremely stable in the injection solvent for up to 32 h. This was a positive aspect of the butylation procedure since with automation which allows 'overnight' sequential analyses, the derivatised sample may not be analysed for up to 24 h following its preparation.

Butyl drug derivatives were chromatographed on both the narrow and wider bore columns. Some differences were noted in the peak profiles and identities of derivatives for a number of drugs (Section 4.3.2.3*i*). As indicated in Appendices

A.4 and B.3.2.2, gas flows through the narrow and wider bore columns differed, with the greater flow through the wider bore column. Consequently, analytes chromatographed on the wider bore column eluted more quickly (demonstrated by RI data in Table 4.6) and were exposed to lower oven temperatures for shorter periods of time prior to elution, than on the narrow bore column. In addition, at the time of injection, there was a greater flow through the injection chamber flushing the volatilised sample more quickly onto the front of the wider bore column. Therefore, the observed differences between the two columns for a particular drug resulted from temperature conditions experienced by derivatives during their chromatography.

For example, the late eluting peak identified on the wider bore column as tri-butyl frusemide was absent on the narrow bore column for one of the following reasons:-

1. the derivative had an RI value > 3400 on the narrow bore column and consequently, was not eluted before the end of the GC run;
2. the derivative completely decomposed following prolonged exposure to excessive heat during its transit through the column; or
3. the derivative completely decomposed in the injector as it was flushed more slowly onto the front of the column.

It is likely that bis- derivatives of desmethylclobazam, ketoprofen, phenytoin and salicylic acid, absent on the narrow bore column, were detected on the wider bore column for similar reasons. It is postulated that, although these derivatives were formed during the alkylation reaction, they were not detected following chromatography on the narrow bore column as they were completely decomposed by their prolonged exposure to excessive temperatures during either splitless injection or column elution.

The peak profile for lorazepam derivatives was complicated and differed between the two columns. A different lorazepam derivative (RI 3051) was chromatographed on the wider bore column and the derivative that was detected on the narrow bore column (RI 2977) was chromatographed as multiple unresolved peaks on the wider bore column. The lorazepam GC artefact detected on the narrow bore column was absent on the wider bore column. This suggests that with chromatography on the narrow bore column, lorazepam derivatives detected on the wider bore column were transformed in the hot injector to thermal decomposition products. It is difficult to ascertain which of the products chromatographed on the narrow bore column related to those chromatographed on the wider bore column without further investigation, but it is postulated that the multiple unresolved peaks detected on the wider bore column were decomposed to a single product (RI 2977) on the narrow bore column. Similarly, the GC artefact detected on the narrow bore column may be related to the derivative (RI 3051) detected on the wider bore column. The decomposition in the injector of products detected only on the narrow bore column from derivatives chromatographed on the wider bore column may also explain the different GC peak profiles observed between columns for allopurinol and oxazepam (Section 4.3.2.3*i*).

Bis- derivatives of chlorpropamide and tolbutamide GC artefacts detected on the

wider bore column were either absent or of negligible peak height on the narrow bore column. In Section 5.3.2.3 it is shown that the GC artefact of underivatised tolbutamide (4-methyl-benzenesulphonamide) was formed in the acylation reaction medium and subsequently derivatised. It is postulated that, in the strongly alkaline butylation reaction medium, the sulphonamide artefacts of both tolbutamide and chlorpropamide are similarly formed and subsequently butylated. It is not clear whether both mono- and bis- derivatives of the artefacts are formed in the reaction medium or whether the mono- derivative arises from the bis-derivative following its thermal decomposition in the injector. It is apparent, however, that the conversion of the underivatised drug to the artefact was incomplete as mono-butyl derivatives of the underivatised drugs (less terminal groups) were detected.

It is likely, therefore, that other sulphonamide nucleophiles (eg. diuretic agents) also possess properties which allow them to readily displace I^- from *n*-butyl iodide and form butyl derivatives in the reaction medium, but these derivatives were not thermally stable in the injector and related products were detected (Tables 4.1 and 4.4). Similarly, other products related to butyl derivatives (eg. the hydrolysis product of sulindac, the related products of benzoylecgonine and captopril, artefact of indomethacin) were also formed in the hot injector.

Data in Section 4.3.2.3*i* indicated a difference in the major derivative which was chromatographed on the narrow and wider bore columns for some drugs with multi-peak profiles. These differences too, can be explained by the difference in temperature and temperature exposure experienced by temperature-sensitive derivatives either in the injector or during column elution. Clearly, the wider bore column was the most appropriate column on which to analyse the butyl derivatives of a wide range of drugs.

Data in Table 4.5 indicate the reproducibility of derivative formation determined on the narrow bore column for sample replicates derivatised on the same day, and on consecutive days. Although the reproducibility tests compared only single samples within and between days, the data did give an indication of derivatives that were formed very reproducibly or not. It was indicated in Section 1.3 that one of the needs to be met by a comprehensive drug screening procedure was to extract and chromatograph the majority of target drugs well. Consequently, in the design of a suitable broad butylation procedure, development was not tailored to an individual drug but designed for a screening technique which would accommodate a large number of acidic drugs. As such, exhaustive studies regarding reproducibility of derivative formation for each of the target drugs were not warranted or possible within the time span of the project. Rather, it was necessary to determine whether butylation of a drug was promising enough for subsequent extraction experiments with target drugs (Chapter 7). It should be noted, however, that as only single samples were compared within and between days it is possible that further experimentation would have revealed that either R1 or S represented 'out-lying' samples.

The butyl derivatives for the majority of drugs tested were formed relatively

reproducibly. For example, the barbiturates and carboxylic acids were reproducibly formed both within and between days. These drugs were also shown to have reactive nucleophiles and high rates of reaction (Table 4.2). However, in general, data in Table 4.5 are difficult to interpret without an indication of the steric influences acting on, and the nucleophilicity of, a given nucleophile which affected the ease with which the butyl derivative was formed (S_N2 reaction).

Clearly some drugs were very variable in their derivative formation particularly between days (eg. allopurinol, benzoylecgonine, the hydrolysis product of sulindac, mono-butyl diflunisal, warfarin, clonazepam, chlorpropamide, paracetamol). It has already been indicated that some butyl drug derivatives are temperature-sensitive. It is postulated, therefore, that in addition to factors associated with individual nucleophiles, temperature-related factors during chromatography were contributing to the apparent variability in derivative formation seen in Table 4.5. It is possible that for the thermally unstable butyl derivatives, the proportion chromatographed as a given derivative was dependent on reactions occurring within the injector and some of these reactions may not have been reproducible. For example, the butylated artefacts of chlorpropamide and tolbutamide were formed in the hot injector and despite structural similarities, tolbutamide was far more reproducibly formed than was chlorpropamide. The structural features of butyl derivatives that convey thermal stability are unknown.

It should be noted that during experiments to optimise the reaction conditions (Table 4.3) some of the observed changes in the peak heights of butyl derivatives may have been due to either the non-reproducible formation of the derivative in the reaction medium, or non-reproducible reactions within the hot injector, rather than in response to an altered reaction condition variable.

It was hoped that, in maximising the chromatographic response of polar drugs through derivatisation, detection at concentrations equivalent to therapeutic blood levels and below would be achieved (Section 4.1). In Section 3.4, it was noted that the chromatographic response for any drug (or derivative) would be maximised when all the injected drug passed unchanged through the separating column to the detector in a narrow, focussed band. Five circumstances were discussed which influenced the chromatographic response. Clearly, for temperature-sensitive derivatives, the maximum chromatographic response was achieved on the wider bore column.

A sixth circumstance which is applicable only to derivatised drugs is that the chromatographic response of a polar drug is maximised when a single non-polar derivative is formed from that drug which passes through the column unchanged. Consequently, chromatographic response of drug derivatives is influenced by:-

1. the number of derivatives formed from a drug;
2. the reproducibility of derivative formation;
3. the stability of the derivative to temperatures experienced during chromatography; and
4. residual polarity of the derivative as determined by its drug structure (Section 3.3.1).

Data in Table 4.3 showed that, for some drugs, the reaction conditions were such that more than one derivative of significant peak height was formed (allopurinol, benzoylecgonine, captopril, diflunisal, lorazepam, oxazepam, phenytoin, primidone, salicylic acid). Consequently, instrument sensitivity to these derivatives would be reduced as each derivative represented some portion of the total drug present. Data in Table 4.9 indicated the detection limits determined on narrow and wider bore columns for many butyl derivatives. For each of the drugs where more than one derivative of significant peak height was formed, the lowest amount detected was relatively high compared with drugs for which a single significant derivative was formed.

Data in Table 4.8 indicated that, although the linearity (reflected by 10^M values) of some butyl derivatives over the concentration range tested was improved on the wider bore column, for the majority of butyl drug derivatives, linearity was not significantly affected by chromatography on either the narrow or wider bore columns. Similarly, good peak shapes were observed for the majority of derivatives on both columns (Table 4.8; A1–A2). Both linearity over a broad concentration range and very good peak shape for a large number of butyl derivatives indicated that, through butylation, the polar functional groups of these drugs were effectively removed, minimising undesirable interaction with active sites on the column and excessive interaction with the stationary phase. Consequently, the chromatographic response of these polar drugs has been either significantly improved or maximised from that observed following their chromatography as underivatised drugs (Tables 3.1–3.4).

As was seen for underivatised drugs (Tables 3.2 and 3.4), log transformed data points for all butyl derivatives were fit by regression equations; however, a curved relationship often described the untransformed data. Picogram to nanogram concentrations were monitored to determine both the concentration range for which a single linear equation would apply, and detection limits for all butyl derivatives. Untransformed data points covering a 100- to 1000-fold increase in concentration were best described by either a linear or a slightly curved relationship for approximately 85% of butyl derivatives tested.

In practice, a concentration curve for a given drug would cover concentrations ranging from sub-therapeutic to high therapeutic levels and a ten-fold increase in concentration would be common. When the concentration range is extended (picogram to nanogram) the relationship observed at higher concentrations may not mirror that observed at low concentrations as adsorption of the drug to active sites on the GC column may be significant at low concentrations. Although a single curve is fitted to the data points in Table 4.8, two or even three linear equations with different gradients could also be fitted to regions of the curve which cover a narrower concentration range particularly where 1000-fold differences in concentration were investigated. Consequently, although the linearity of a derivative may be described by a power equation ($M < 20$) over an extended concentration range, over a restricted range, the majority of derivatives would be linear.

For 15% of butyl derivatives, three data points which covered a ten-fold increase in concentration were best fitted by a curve. Three data points are likely to be inadequate to accurately define the curve and for some, the data points were not well fitted (r^2 from 0.962 to 0.980). Clearly, these derivatives were not linear even within the practical concentration range. This may have resulted from non-reproducible derivatisation, non-reproducible thermal decomposition of derivatives, or the fact that a significant degree of polarity was retained by the derivatised drug. Butylation will only remove polar functional groups which react with *n*-butyl iodide. If a drug also possesses polar functional groups not susceptible to alkylation, these functions will continue to interact undesirably with the column and lead to irreversible adsorption and asymmetric peak shapes.

For the majority (85%) of butyl derivatives tested on the two columns, either a similar, or increased instrument sensitivity was observed (Table 4.9). These data are comparable with data in Table B.12 for underivatised drugs. However, for 47% of underivatised test drugs, but only 20% of butyl derivatives, an increased chromatographic response was observed on the wider bore column from that on the narrow bore column. This difference is thought to have occurred due to differences in the apparent 'polarity' of drugs (Section 3.3.1). The butyl derivatives are significantly less polar than the underivatised drugs from which they were formed. Many of these underivatised drugs were so polar that they could not be chromatographed (eg carboxylic acids - Tables 3.1–3.4). The butyl derivatives, however, still retain some of the polarity imparted to them by their drug structure. The residual polarity allows interaction with active sites on the column resulting in adsorption which becomes most apparent at low concentrations. Consequently, at the critical threshold of instrument detection, any chromatographic advantage conveyed following chromatography on the wider bore column was obscured by column adsorption and no effective decrease in instrument detection was observed. It should be noted that following chromatography on both the narrow and wider bore columns, instrument sensitivity for 46% of underivatised drugs was also similar. Consequently, the polar nature of a drug or drug derivative, though it be weak (reflected by good peak shape and linearity), has a significant influence on the observed detection limits. The residual polarity of derivatives which results in loss of the injected sample through column adsorption also affects the injected range over which chromatography is linear and power equations are observed (Sections 5.4.2.2–5.4.2.4).

The chromatographic characteristics of a wide range of acidic drug derivatives across different drug groups was investigated following splitless injection using both narrow and wider bore columns. The chromatographic information recorded establishes a database of observed behaviour for these derivatives on a non-polar chromatographic system (eg. RI, peak shape, linearity over a defined concentration range, limit of instrument detection).

It was indicated in Section 4.3.2 that the benzodiazepine structure allows the possibility that tautomeric structures form. The same can occur with the barbiturate structure. Bis- derivatives were always identified for barbiturates,

however, while isomers of a mono- derivative were identified for benzodiazepines. The barbiturates possess two acidic imide groups each of which can be alkylated at either the N or O atom. Through a combination of resonance effects and solvation, the attacking atom is nitrogen rather than oxygen, and bis-derivatives are formed.[7] In contrast, the benzodiazepines produced two mono-butyl isomers resulting from either an attacking O or N atom. It is thought that under the present reaction conditions, like the barbiturates, the nucleophile with an attacking O atom was substantially less reactive than the nucleophile with an attacking N atom and so only one mono- butyl isomer was formed predominantly.

Chromatography of many of the benzodiazepine drugs was not linear over the concentration range tested (Table 4.8) and the 'lowest' amount detected (Table 4.9) was unrealistically high for detection of these drugs at therapeutic levels in biological samples. Table 4.9 indicated that, following their chromatography on the wider bore column, the butyl derivatives of desmethyl metabolites of the benzodiazepines tested had relatively low detection limits compared with either the underivatised drugs or derivatives of the 7-amino metabolite. Consequently, although butylation of benzodiazepines with *n*-butyl iodide was possible as reported[1,5,8] and a single significant derivative was formed (unlike methylation with diazomethane), the procedure was not suitable for quantitative work except with the desmethyl metabolites. Similarly, butylation of morphine with *n*-butyl iodide under the current reaction conditions was not ideal as morphine showed both a high detection limit and the poorest linearity of any derivative tested.

References

1. "Alkylation with alkyl halides as a derivatisation method for the gas chromatographic determination of acidic pharmaceuticals."
Hulshoff A, Förch A.D; J Chromatogr. (1981) 220, 275-311

2. "Derivative formation in the quantitative gas-chromatographic analysis of pharmaceuticals. A review"
Nicholson J D; The Analyst (1978) 103, 1-28

3. "Chromatography Today."
Poole C, Poole S (Eds.); 1st Edition (1991) Elsevier Scientific Publishing Company Inc, New York; 848-866

4. "Mass spectral and GC data of drugs, poisons and their metabolites."
Pfleger K, Mauer H, Weber A (Eds.) 1st Edition (1985) Zechnersche Buchdruckerei, Federal Republic of Germany.

5. "Derivatisation reactions in the gas-liquid chromatographic analysis of drugs in biological fluids."
Hulshoff A, Lingeman H; J Pharmaceutical & Biomed Analysis (1984) 2,337-380

6. "Sensitive gas chromatographic method for the determination of diazepam and n-desmethyl diazepam in plasma."
De Gier J J, 't Hart B J; J Chromatogr. (1979) 163, 304-309

7. "Determination of intact oxazepam by electron capture gas chromatography after extractive alkylation reaction."
Vessman J, Johansson M, Magnusson P; Anal. Chem. (1977) 49, 1545-1549

8. "Quaternary ammonium salts for butylation and mass spectral identification of volatile organic acids."
Burke D G, Halpern B; Anal. Chem. (1983) 55, 822-826

9. "Rapid and simple clean-up and derivatisation procedure for the gas chromatographic determination of acidic drugs in plasma."
Roseboom H, Hulshoff A; J Chromatogr. (1979) 173,65-74

10. "Rapid esterification for gas chromatography."
Greenley R H; J Chromatogr. (1974) 88,229-233

11. "Advanced Organic Chemistry - Reacion Mechanisms and Structure."
March J(Ed); 1992, 348 - 362; 4th edition, John Wiley and Sons Inc USA.

12. "Practical Organic Chemistry."
Vogel A (Ed.) 1st Edition (1956) Longmans, London, 576

13. "Decomposition of benzodiazepines during analysis of capillary gas chromatography-mass spectrometry."
Joyce J, Bal T, Ardrey R, Stevens H, Moffat A; Biomedical Mass Spectrometry (1984) 6, 284 - 289

14. "Mechanism and structure in organic chemistry."
Gould E S; 1959, 250 - 280; Holt, Rhinhart and Winston Inc. USA.

Chapter 5.

ACYLATION AND CHROMATOGRAPHY OF PENTAFLUOROPROPIONYL DERIVATIVES

5.1 Introduction

5.1.1 General

Tables 3.2 and 3.4 showed quantitative chromatographic data for underivatised drugs. From these data it was apparent that many basic drugs (eg. amines) were too polar to chromatograph well and, consequently, these drugs were either not chromatographed at all, or were chromatographed as broad, tailing peaks which possessed poor linearity and relatively high instrument detection limits. It was shown in Chapter 4 that derivatisation of acidic drugs with non-polar butyl group/s significantly improved the chromatographic characteristics of the majority of drugs investigated. For example, the chromatography of drugs with carboxylic acid functions were so improved following their conversion to butyl derivatives that a GC peak was chromatographed with 'Gaussian' peak shapes where none was detected for the underivatised drugs. Butylation, however, is not suitable for the derivatisation of basic drugs.

Acylation is possible where:-

1. the polar functional group on the drug has an acidic proton which can be lost; and
2. the resulting conjugate base has suitable nucleophilic properties which cause it to react with the acylating reagent.

Table 5.1 shows the polar functional groups capable of participating in acylation reactions.[1-3] The majority of drugs selected for investigation possessed either amine (1° or 2°) or hydroxyl (alcohol or phenol) functions.

Acylation developmental work was performed on the wider bore (0.32 mm i.d.) capillary column following both on-column (OC) and splitless injection. It was hoped that in maximising the chromatographic response of polar basic drugs through derivatisation, detection at concentrations equivalent to therapeutic blood levels and below would be achieved.

5.1.2 The Reaction

Acylation reactions involve reagents which contain an acyl group - a carbonyl group joined to an oxygen, nitrogen, halogen, sulphur or other electronegative atom.[4,5] The acylation reaction is similar to that of alkylation reactions in that a nucleophile (the deprotonated drug) displaces a leaving group (eg. a carboxylate ion from a carboxylic acid anhydride) from the derivatising reagent in a nucleophilic substitution reaction (Section 4.1.2). The acylation reaction, however, does not proceed via a S_N2 mechanism as do alkylation reactions. The reaction mechanism is discussed in Appendix C.1.

5.1.3 The Acylating Reagent

As indicated in Appendix C.1, acylation reactions involve reagents containing a carbonyl group joined to an electronegative atom and the most reactive of these are acyl chlorides or anhydrides. In early GC studies acetyl and other aliphatic reagents were used which produced derivatives with increased molecular weight, volatility and retention time.[2,6] The corresponding fluorinated derivatives, however, proved to be more versatile as they produced derivatives of greater volatility (shorter retention times) and increased sensitivity for electron capture detectors.[2,6] The advantages and disadvantages of employing different fluorinated reagents are discussed in Appendix C.2.

Pentafluoropropionic anhydride (PFPA) was selected as the acylating reagent in the following experiments due to its greater volatility and the stability and shorter retention times of PFP derivatives (lower temperature of analysis) compared with other fluorinated acylating reagents (Appendix C.2)[1]

5.1.4 Reaction Catalysts

Acid- and base-catalysed mechanisms determine the rates of formation and decomposition of the tetrahedral intermediate (Appendix C.1).[5] Reaction catalysts are discussed in Appendix C.3.

In general, catalysis by a base or basic solvent promotes the acylation of compounds and in the selection of a catalyst practical factors such as the following must be considered:-

1. the subsequent removal of the catalyst,
2. its chromatographic properties if not completely removed,
3. formation of additional products through side reactions,
4. its ability to catalyse reactions with different nucleophiles (eg drugs with different functional groups) and
5. whether or not reaction conditions with a single catalyst can be optimised for the acylation of drugs with different functional groups.

5.2 Methods

Refer to "General Procedure" (Chapter 2). In particular, for HC marker solution refer to Section 2.2.5.5 and 2.2.5.6; for reference drug solutions refer to Section 2.2.5.14; For analyses with the Hewlett Packard GC/MSD refer to:-

Section 2.4.1 for instrumentation;
Section 2.4.3.1 for SIM acquisition parameters;
Section 2.4.2 and Appendix B.2.2 for gas chromatographic parameters with the wider bore column employing splitless and on-column injection techniques respectively.

Qualitative and quantitative chromatographic data for drug derivatives was obtained using the method described in Section 2.6.1 unless otherwise specified.

5.2.1 Instrumentation

Chemical ionisation (CI) and high resolution electron impact mass spectrometry

analyses were performed on the Kratos Concept ISQ magnetic mass spectrometer with direct insertion probe (DIP) into the ion source at either 140°C (cool source) or 220°C (hot source). Chemical ionisation was achieved with either ammonia or methane gas.

Experiments which compared splitless injection (fixed temperature) with programmable temperature injection employed the Varian 1094 liquid CO_2 cooled septum-equipped temperature programmable injector (SPI). A Varian 3400 gas chromatograph with both splitless (Varian 1077) and SPI injectors was coupled with a Varian Saturn II ion trap detector. There was a direct capillary interface to the detector and it was operated in full scan mode by PC-based Saturn Version 5.1 operating software.

5.2.2. Gas Chromatography

The SPI analyses on the Varian Saturn II used a borosilicate glass, high performance liner without packing; 40°C (held for 0.2 min) to 320°C at 180 °C/min; 1 μL splitless automated injection. Automated split/splitless injection at 260°C used a borosilicate glass liner with frit. A 30 m J&W high performance capillary column (DB-5MS) with 0.25 mm i.d. and 0.10μm film thickness was used with all analyses. The carrier gas was helium (10 psi) and 1 μL splitless injections were made with the split closed for 0.7 min. Temperature parameters were as follows:-

transfer line	290°C	
oven initial	50°C	hold time = 1 min
final	320°C	hold time = 9 min
temp. rate	10°C/min	

Refer to Appendix B.2.2 for electronic pressure controlled (EPC) analyses on the Kratos instrument. Head pressures of 10 and 40 psi were used. Ten psi approximated the conditions for splitless injection on the low resolution benchtop GC/MS. Following injection, a column head pressure of 15 psi was established for the duration of the run.

5.2.3 Derivatisation with PFPA

Refer to Section 2.2.4 for details of reagents and to Section 2.3.2 for apparatus employed; refer to Figure 2.5 for the derivatisation procedure which was performed without the 'azeotrope formation' step (Section 9.4.2). During development of the derivatisation procedure, variation to some parameters included the following:-

1. volume of PFPA:- 2, 25, 50 and 100 μL
2. time of reaction:- 5, 15, 30, 60 and 120 min
3. use of catalysts:- 3, 6 and 9 μL pyridine (over anhydrous sodium sulphate)
   2, 5 and 10 μL diethylamine (DEA)
   50 μL acetonitrile

5.3 Results

5.3.1 PFPA Reaction Conditions

Preliminary experiments with PFPA used four drugs of abuse which each possessed

functional groups susceptible to acylation as follows:-

amphetamine - 1° amine;
methylamphetamine - 2° amine;
morphine and codeine - alcohols.

The GC peaks detected were identified by their low resolution EI full-scan mass spectra as the mono-PFP derivatives of these drugs (either M^+ + *m/z* 147 or a characteristic fragment *m/z* ion + *m/z* 147; where *m/z* 147 = the pentafluoropropionyl group $-C_3F_5O$).

Different volumes of PFPA (Section 5.2.3) were added to reaction vials which contained the dried residue of the test drugs (approximately 80 ng) and the GC peak areas of the derivatives were monitored. Initially PFPA alone, or in equal volumes with either toluene or ethyl acetate, was reacted at 85°C in a heating block for 20 min.

The greatest peak area for each derivative was obtained in the presence of ethyl acetate and similar peak areas were obtained with either PFPA alone or PFPA and toluene. When 25, 50 or 100 µL volumes of PFPA were reacted with the drugs, the peak areas of derivatives were similar but all were smaller when 2 µL was used.

5.3.1.1 *Catalysis*

Amphetamine, methylamphetamine, morphine and codeine were used in a test mix to investigate whether or not the acylating properties of PFPA were enhanced in the presence of strong basic catalysts (pyridine and DEA). Ethyl acetate was added to the reaction mixture. Each catalyst was used in the approximate molar ratio to PFPA (10 µL) cited in the literature.[1,7-12]

In the presence of either catalyst the following was observed.

1. A strongly yellow coloured reaction mixture resulted whenever morphine or codeine was derivatised in the presence of either catalyst but was most apparent with DEA. The discolouration increased with the amount of catalyst added.
2. The reaction mixture was difficult to evaporate under nitrogen and required heating. DEA was difficult to completely remove without use of excessive heat. After drying whenever pyridine was used, a large amount of residue remained which was soluble in the injection solvent ($CHCl_3$).
3. In chromatograms produced following the reaction, additional peaks were detected which eluted in the region of the amphetamine derivatives and interfered particularly with *m/z* 91.05 and *m/z* 118.05.
4. The effects of catalysts on the peak areas of the amphetamine derivatives were minimal, but they greatly reduced the peak areas of the opiate derivatives. For example, when pyridine was added at ≥ 6 µL the peak area of mono-PFP morphine decreased from that at 3 µL or when no pyridine was present.
5. Unless the catalysts were dried, vapours were generated when the PFPA was added to the reaction vial and the peak areas of all derivatives were reduced or absent.

Acetonitrile both solubilises the drug residue and, as a basic solvent, acts as a weak basic catalyst (Section 5.1.4). The following was observed when PFPA was used in equal volume with acetonitrile.

1. There was no discolouration of the reaction mixture.
2. The reaction mixture was easily removed under nitrogen without any additional residue formation.
3. The chromatograms produced were similar to those obtained with PFPA/ethyl acetate and no additional peaks were detected.
4. The peak areas of the opiate derivatives were not reduced.
5. The reproducibility and peak area of the codeine derivative increased when compared with that obtained with a PFPA/ethyl acetate reaction mixture.

In all subsequent experiments, PFPA (50 µL) and acetonitrile (50 µL) were added to the dried residues of test drugs and reacted at 85°C. On completion, the reaction mixture was removed under nitrogen and the residue solubilised in either ethyl acetate or the ethyl acetate GC injection solvent (Section 2.2.5.8) prior to GC analysis.

5.3.1.2 *Application to Drugs with Different Functional Groups*

Data in Table 5.2 show the PFP derivatives formed from an expanded number of test drugs possessing different functional groups. Low resolution EI full-scan mass spectra identified GC peaks as either PFP derivatives (mono- and bis-) or related products which contained the PFP group (losses of *m/z* 147 or *m/z* 119; where *m/z* 119 = $-C_2F_5$).

For all drugs except allopurinol, a GC peak was detected which was identified as either a PFP drug derivative or related product. No underivatised drug was detected. Clearly, PFPA reacted with drugs possessing a range of different functional groups although the product of derivatisation was not always immediately apparent from the low resolution EI full-scan mass spectra generated.

5.3.1.3 *Time of Reaction*

Data in Table 5.3 compares the GC peak areas of derivatives formed from some of the drugs tested in Section 5.3.1.2 following reaction with PFPA for 5, 15, 30, 60 and 120 min. Duplicate samples were derivatised and the mean peak area of each derivative expressed as a percentage of that obtained after 60 min (100%).

Derivatisation of codeine, THC, desipramine, methylphenidate, metoclopramide, and propranolol was rapid (5 min) and no further increase in the peak area of the derivative was observed with increased reaction time. Extended reaction times (> 5 min) did not reduce the peak areas of these derivatives.

The peak areas of amphetamine, lorazepam, and terfenadine derivatives increased with increasing reaction time up to 120 min. The peak areas of these derivatives (particularly lorazepam and terfenadine) may have increased further with longer reaction times (> 120 min). The peak areas of replicates obtained for derivatives of oxyphenbutazone and cimetidine were so variable that no data could be obtained.

The variability between replicates may indicate that either drug derivatisation or derivative chromatography was non-reproducible. In contrast, the peak areas of the 60 min replicates for the remaining drugs (except lorazepam and terfenadine) were very similar.

Following a reaction time of 60 min, all the drugs tested (except lorazepam and terfenadine) had formed a derivative with either the maximum or close to the maximum peak area. In all subsequent derivatisation reactions with PFPA, the reaction time was 60 min.

5.3.2 Acylation of Drug Standards

5.3.2.1 *Identification of PFP Derivatives*

Approximately 45 test drugs with functional groups potentially suitable for acylation (Table 5.1) were derivatised with PFPA. The majority of derivatives were identified through low resolution EI full-scan mass spectrometry as mono- and bis- derivatives (Section 5.3.2.4). The GC peaks detected following derivatisation of 22 of these drugs, however, could not be unequivocally identified although their mass spectra indicated that each contained PFP group/s. Table 5.4 shows the chemical ionisation (CI) and high and low resolution EI mass spectral data obtained for these derivatisation products.

For many of the drugs, multiple products were formed following their reaction with PFPA (Table 5.4). In most cases, mass spectral analyses (CI and high resolution EI) with the direct insertion probe (DIP) were able to differentiate between different products as they distilled from the tip of the probe. Where sufficient resolution of products was not possible, the sample was introduced to the source via gas chromatography.

The probable structure for the majority of derivatisation products was determined from the CI mass spectrum of the product and the known underivatised drug structure. For some derivatisation products, however, the probable structure was still not clear and high resolution EI mass spectral data was obtained which accurately determined the atomic mass of the derivative and/or its fragment *m/z* ions. The most logical empirical formula ($\leq$ 2 ppm) generated by the Kratos peak matching program on the basis of the accurate mass determination is listed for these drugs.

CI was used to determine the M^+ (as H^+ or NH_4^+ adducts) of the derivatisation product since, in low resolution EI mass spectra, M^+ is often absent. Data in Table 5.4 show that, in many instances, the determined M^+ corresponded to either the mono- or bis-PFP derivative of the drug and the identity and site of derivatisation was often confirmed by the probable structures determined for EI spectral fragments of the derivative (clonidine, hyoscyamine, lorazepam, methylphenidate, nifedipine, oxyphenbutazone, procainamide, propranolol, sulphamethoxazole, timolol, warfarin). For pindolol, however, the data produced from both ammonia and methane CI mass spectrometry was inconclusive. The low resolution EI mass spectra were identical for three well resolved GC peaks and indicated the formation of three bis- derivatives, yet no higher mass (M^+) was apparent for any peak by CI

mass spectrometry. This could not be explained.

Data in Table 5.4 indicate that unexpected products were formed from some drugs reacted with PFPA through chemical reactions/rearrangements within the reaction mixture. Dehydrated underivatised drugs and dehydrated drugs acylated at other sites were the most commonly occurring of these unusual derivatisation products (disopyramide, etafedrine, haloperidol, hyoscyamine, labetolol, procainamide, terfenadine, warfarin). Labetolol and terfenadine were both doubly dehydrated during the reaction.

Some drugs produced unique PFP derivatives which were not formed by other structurally-related drugs. In the majority of cases, products formed from these drugs in the reaction medium were subsequently acylated to PFP derivatives as follows.

1. Desipramine, formed a bis-PFP derivative which was not predicted from the underivatised drug structure in a chemical process not encountered during the derivatisation of any other 2° amine.
2. Labetalol, unlike any other β-blocker tested, formed the bis- derivative of the doubly dehydrated drug.
3. A chemical rearrangement during the formation of the bis-PFP derivative of captopril resulted in the expulsion of -COOH, unlike any other drug derivatised at its carboxylic acid function.

Data in Table 5.4 show that the presence of the PFP group on some drugs resulted in EI mass spectral fragmentation patterns unlike those of the underivatised drugs. Through unusual rearrangements resulting in the expulsion of groups from apparently stable structures, spectral fragments were formed with structures which were difficult to identify without accurate mass data and peak matching (cimetidine, lorazepam, nifedipine, sulphamethoxazole, tolbutamide).

5.3.2.2 *Effect of Injection Technique on the Chromatography of PFP Derivatives.*

During experiments identifying PFP derivatives (Section 5.3.2.1) it was observed that the PFP-related products detected for some drugs (etafedrine, nifedipine, procainamide, timolol) by GC-MS (splitless injection at 260°C) were absent during DIP analyses (ion source at 200°C). It was postulated that the PFP derivatives formed in the reaction medium were altered/decomposed during their GC analysis.

The effect of injection temperature on the peak areas of test PFP derivatives was examined with three different injection techniques: electronic pressure controlled injection (EPC), septum-equipped programmable injection (SPI) and on-column (OC) injection. Through EPC injection, derivative exposure to an elevated injector temperature would be reduced. Through SPI, each derivative would be exposed to the minimum temperature required to volatilise it. Through OC injection, exposure to an elevated injection temperature would be removed (Appendix B.4.1.2). The test PFP derivatives were a mixture of mono- and bis-PFP derivatives, dehydrated drugs (underivatised and derivatised) and 'GC artefacts' - products thought to be formed in

the hot injector (eg. etafedrine and timolol).

Refer to Section 5.2 for details of instrumentation and chromatographic parameters. In brief, however, EPC injection (260°C; splitless) was made at column head pressures of 10 and 40 psi where 10 psi approximated 'normal' splitless injection; SPI injection ramping from 40 to 320°C at 180°C/min was compared with splitless injection (260°C) on the same instrument at the same column head pressure; OC injection was compared with splitless injection (260°C) on the same instrument at the same column head pressure.

Data in Table 5.5 show the change in peak area of a given PFP derivative following each of the three injection techniques as a percentage of that obtained following splitless injection on that instrument. The peak areas obtained with each injection technique for a given derivative could not be directly compared as chromatographic variables and instrumentation differed. Similarly, although the same test drug solution was used to investigate the three injection techniques, it was derivatised on one of three different days and without information relating to the reproducibility of PFP derivative formation, the data was not directly comparable. It should be noted that the splitless injection for comparison with SPI injection was not optimised on that instrument and, consequently, peak area differences were exaggerated by poor chromatography of the derivatives following splitless injection. Trends in the chromatographic behaviour of the PFP derivatives following each of the three different injection techniques were apparent (Table 5.5).

Data in Table 5.5 indicate the following.

1. The peak areas for the majority of test PFP derivatives increased following injection by all three injection techniques from those obtained following splitless injection (> 100% or +).
2. The increase in derivative peak area from that obtained with splitless injection on the same instrument appeared greater for SPI and OC injections than for EPC injections as, for SPI and OC injections, no GC peak had been detected following the corresponding splitless injections (cimetidine, hyoscine, pholcodine, bis-derivatives of clonidine and procainamide).
3. Mono-PFP hyoscyamine was chromatographed only following OC injection.
4. Mono-PFP etafedrine was chromatographed as a single peak following OC and SPI injections, but following EPC and splitless injections it was chromatographed as several unresolved peaks.
5. The artefacts of both cimetidine and timolol were reduced or absent following OC injection, confirming initial data (Table 5.4) which indicated they were formed in the hot injector (GC artefacts) rather than through reaction of the drug with PFPA.
6. The peak area of the artefact of timolol was reduced by all three injection techniques but that of cimetidine was reduced only following OC injection.
7. Where drugs were associated with dehydrated products (derivatised and underivatised) all except procainamide were detected following all three injection techniques.

These data suggest that as dehydrated drug products were present even following OC injection they were formed through dehydration of the drug by PFPA in the reaction medium prior to GC analysis (reaction artefacts). In contrast, dehydrated procainamide was absent following OC injection indicating that it was formed in the hot injector as a GC artefact. These data indicate that, in general, the peak areas of reaction artefacts were unaffected by the injection technique, but that of the mono-PFP derivative of dehydrated haloperidol was reduced following splitless injection.

The data in Table 5.5 indicate that splitless injection at 260°C diminished the peak areas for this group of test PFP derivatives except where the peak was a GC artefact or a reaction artefact (except for haloperidol). Following OC injection, only derivatives formed from the reaction of drug with PFPA were chromatographed and related GC artefacts were absent.

5.3.2.3 *On-column and Splitless Injections*

Data in Table 5.5 showed that mono-PFP derivatives of etafedrine, hyoscine and hyoscyamine were only detected following OC injection. It was postulated, therefore that, following OC rather than splitless injection, different PFP derivatives of other drugs may be detected.

Table 5.6 lists the presence or absence of a GC peak following OC and splitless injections for some PFPA derivatised drugs. The characteristic *m/z* ions from their low resolution EI mass spectra are shown for each derivative/related product. No characteristic *m/z* ions are listed where multiple peaks (M) or broad peaks with changing ion profiles (R) were chromatographed. In some cases, a comment on the chromatographic characteristics of a particular GC peak is given. Similarly, conclusions are listed with regard to how/where a particular drug derivative or related product was formed (reaction medium or GC artefact). Where the same peak was detected following either injection technique, the peak area obtained following splitless injection was compared with that following OC injection (>, < and ≅). In some cases the peak areas of derivatisation products varied following multiple injections of the same type (↑↓).

Data in Table 5.6 indicate the following.

1. The chromatography of some PFP drug derivatives was unaffected by injection technique as their GC peak areas were similar following both OC and splitless injections.
2. The detection and identity of some PFP drug derivatives was dependent on the injection technique employed.
3. Some drugs were not suitable for derivatisation with PFPA, as following each injection technique either no GC peak or multiple peaks were detected.

The multiple peaks detected for a given drug had different but related low resolution EI full-scan mass spectra which were difficult to relate to either the drug structure or the low resolution EI full-scan mass spectrum of the underivatised drug. Consequently, the derivatised products for these drugs were not identified.

Following OC injection, some drugs were chromatographed either as broad tailing peaks (T) or as irregularly-shaped complex peaks (R; indicating irregular). The low resolution EI mass spectra of these peaks indicated that they were a mixture of unresolved but related peaks. Following splitless injection, unresolved peaks (T; R) were chromatographed as individual GC peaks with baseline resolution (eg captopril, Figure 5.1). It was concluded that PFP derivatives with 'T' or 'R' characteristics were a mixture of thermally sensitive PFP derivatives and their corresponding degradation artefacts were formed as the derivatives degraded on the column with increasing oven temperature (Section 5.4.2.2).

It was indicated in Tables 5.4 and 5.5 that an elevated injector temperature (splitless injection) could result in the formation of GC artefacts for susceptible derivatives which were absent following analysis by either DIP or OC injection. Underivatised carbamazepine, hyoscyamine and tolbutamide were detected following both OC and splitless injection of an underivatised drug solution (Table 5.6). Following splitless injection, a GC artefact was detected for each underivatised drug which was predictably absent following OC injection. Following derivatisation of the drug solution, the same GC artefacts identified following splitless injection were also detected following OC injection, and those of carbamazepine and tolbutamide were identified as mono-PFP derivatives. These data clearly indicate that these products were not GC artefacts but products formed in the reaction medium and subsequently derivatised.

Data in Table 5.5 suggest that the dehydration of haloperidol, hyoscine and hyoscyamine occurred in the reaction medium and did not result from conditions created in the hot injector. Data in Table 5.6 confirm this and show that other dehydrated drugs identified in Table 5.4 were also formed in the reaction medium (labetolol, warfarin, terfenadine). The dehydrated products of etafedrine and procainamide, however, were GC artefacts.

Unusual derivatisation products detected following both OC and splitless injections indicate that they, too, were reaction artefacts produced following structural rearrangement of the drug in the reaction medium (eg. desipramine, tolbutamide and oxycodone; Table 5.6; 'Comments').

Data in Table 5.6 confirm data in Table 5.5 and show that GC artefacts are produced from derivatisation products of some drugs following their splitless injection (eg. cimetidine, oxazepam).

Data in Table 5.6 indicate that some PFP drug derivatives were detected only following OC injection and the peak areas of others which were obtained following OC injection were greatly reduced following their splitless injection. These data suggest that, although these PFP derivatives were stable at column temperatures (chromatographing as single peaks), they were unstable at the splitless injection temperature and their degradation in the injector led to reduced peak areas. In some cases, the degradation was complete and no peak was detected following splitless injection.

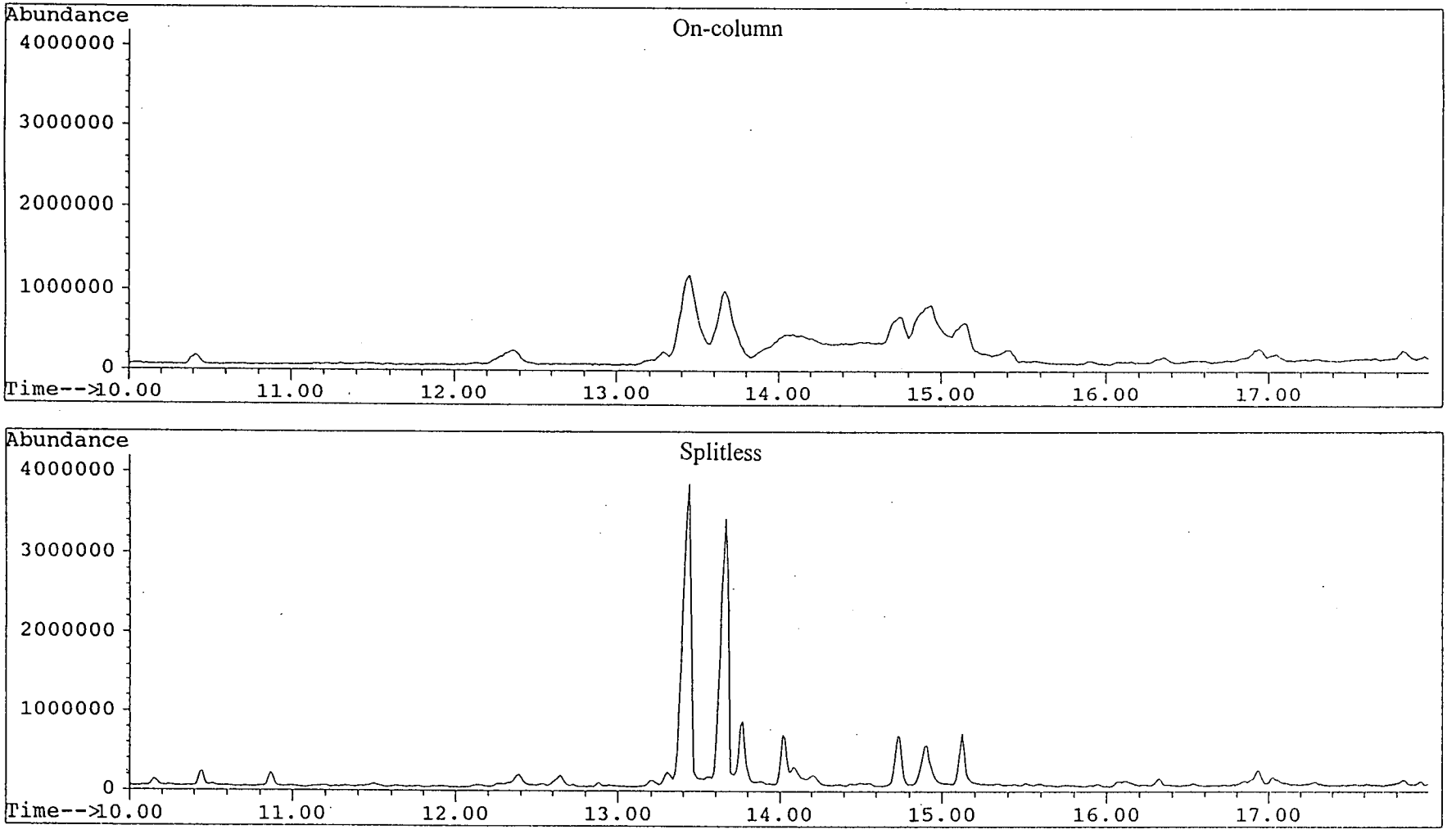


Figure 5.1: Chromatograms obtained following 1 µL on-column and splitless (260°C) injections of PFPA derivatised captopril.

Data in Table 5.6 show that some PFP drug derivatives detected following OC injection were chromatographed as one or more GC peaks with related *m/z* ion profiles following splitless injection (eg etafedrine; Figure 5.2). The GC peak profiles for these drugs were complicated by splitless injection. In contrast, data in Table 5.6 show that the peak profiles of clonidine, hyoscine and hyoscyamine were simplified following splitless injection.

Following both OC and splitless injection for 40 drugs derivatised by PFPA, data in Table 5.6 has:-

1. identified drugs not derivatised by PFPA;
2. shown that an elevated injection temperature can be both detrimental to the stability of PFP derivatives (destroying them), and/or influence the product that is chromatographed (altering them);
3. identified GC artefacts formed from the PFP derivatives of some drugs following their splitless injection;
4. identified some PFP derivatives so temperature sensitive that they degrade into artefacts at oven temperatures; and
5. indicated that dehydration of drugs and other structural changes can occur in the reaction medium resulting in reaction artefacts.

Following identification (including data in Table 5.4) and GC analysis of derivatives formed from approximately 40 test drugs (splitless and OC injections) it was concluded that unusual derivatisation products were generally not formed at a 1° or 2° amine group on a drug but at alcohol or 1° amide groups. Examples of drugs and proposed reactions and products follow, with reference to related previous reports.

1. enol formation[6]	oxycodone	bis-PFP
2. dehydrogenation[13]	desipramine	bis-PFP
3. decarboxylation	captopril	bis-PFP
4. dehydration of 1° amide[6,14]	disopyramide	underivatised (nitrile formation)
5. dehydration of an alcohol	labetolol	bis-PFP (doubly dehydrated)
	warfarin	underivatised
6. deamidation	carbamazepine	PFP and underivatised
	tolbutamide	PFP and underivatised
7. unexplained rearrangements	cimetidine	PFP-cimetidine product
	lorazepam	PFP-lorazepam product

5.3.2.4 *Chromatography of PFP Derivatives*

Qualitative chromatographic data for approximately 90 PFP drug derivatives and related products formed in acylation reactions with PFPA are shown in Table 5.7. The identity of the derivatisation product (when known), characteristic *m/z* ions from low resolution EI mass spectra, and RI (where calculated), are listed. Where more than one derivatisation product was detected following drug acylation, the dominant peak ([1]) is indicated. 'Comments' indicate the functional groups derivatised where polyfunctional drugs were acylated, the formation of either reaction or GC artefacts for some drugs, and thermally sensitive derivatisation products. Structurally-related drugs are grouped under headings which reflect a common chemical structure (eg. barbiturates, benzodiazepines; Figure 3.1), and structurally-unrelated drugs are

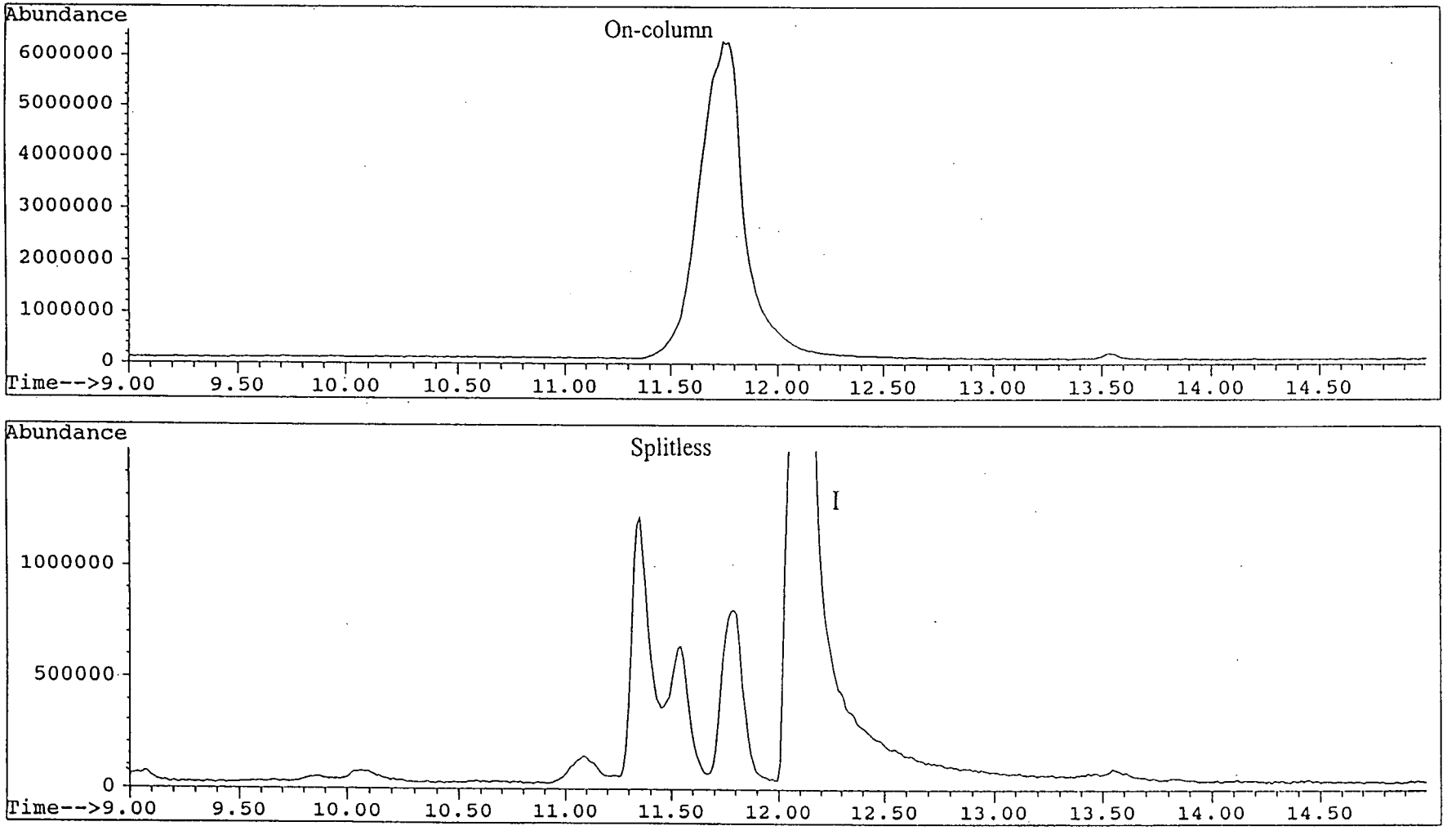


Figure 5.2: Chromatograms obtained following 1 µL on-column and splitless (260°C) injections of PFPA derivatised etafedrine. The chromatogram obtained following splitless injection shows interference (I) from the closely eluting procainamide artefact.

grouped where they possessed a common reactive functional group (eg. 1° amines, alcohols). The drug groupings used in this and the remaining tables in this chapter are discussed in Section 3.3.1.

The data in Table 5.7 show that 17 of the drugs selected for reaction with PFPA did not form a PFP-related product (-). A predominant derivatisation product (PFP derivative, reaction artefact) was chromatographed for 49 drugs which was relatively stable at both oven and injector temperatures. An additional 6 drugs formed a predominant derivatisation product only following OC injection (eg pholcodine, sulphamethoxazole). Following OC injection for 4 drugs, more than one major product of derivatisation was chromatographed (eg procainamide). Following OC and splitless injection more than one product of derivatisation predominated for three drugs (paracetamol, oxycodone, haloperidol). Five drugs formed either single, or multiple (M), derivatisation products which were thermally unstable at oven (eg clonidine, trimethoprim) or injector temperatures (eg chlordiazepoxide). Reaction artefacts (derivatised and underivatised) were formed from 14 drugs following reaction with PFPA. GC artefacts were formed from 5 drugs (splitless injection only).

Data in Table 5.7 show that a predominant PFP derivative was produced for the majority of drugs derivatised at 1° and 2° amine or hydroxyl (alcohol, phenol) groups, and the derivative formed was stable at both oven and injector temperatures. In contrast, the majority of benzodiazepines and drugs derivatised at a sulphonamide (diuretic agents) or sulphonylurea groupings (anti-diabetic agents) formed either no derivative, or derivatives and/or products which were thermally unstable.

Despite these general observations, however, data in Table 5.7 clearly show that not all drugs with common functional groups reacted with PFPA in the same way. The data show that drugs with common alcohol and phenol groups did not react with PFPA in the same way to form derivatives with similar thermal stabilities. Clearly, the product/s of PFPA derivatisation and their thermal stability could not be predicted on the basis of the functional groups derivatised.

The derivatives formed from structurally related drugs (eg. barbiturates, ß-blocking agents, phenylethylamine derivatives) and their chromatographic characteristics were similar. For example, no PFP-related compound was formed by any of the diuretic agents. The anti-diabetic agents each expelled the SO_2 group in their side-chain and permitted PFPA derivatisation of an amine. The ß-blocking agents (except labetalol and sotalol) each produced a bis-PFP derivative with similar low resolution EI mass spectra (*m/z* 366, 408) which differed only in the M^+. Phenylethylamine derivatives all produced thermally stable mono-PFP derivatives.

Data in Table 5.7 indicate that the thermal stability of PFP derivatives ranged from unstable at oven temperatures (severely tailing peaks; eg clonidine) to stable at both oven and injector temperatures. In general, the greatest thermal stability during chromatography was shown by drugs derivatised at amine and hydroxyl groups. The greatest number of PFP derivatives were detected following OC rather than splitless

injection, although the GC peak profile of a drug may have been complicated as multiple derivatives were detected.

i. Stability and Reproducibility

Table 5.8 shows data relating to the reproducibility of derivative formation and their stability with time for some PFP derivatives. The predominant derivative (Table 5.7) was investigated where more than one PFP derivative was formed from a drug. In each case the GC peak area obtained for a given PFP derivative was expressed as a 'peak area ratio' (Section 2.6.1).

The stability data were obtained over 21 h. The GC peak areas of test derivatives (after 11 h and after 16–21 h) were compared with those obtained from a control (0 h; Section 2.6.1). The percentage difference between GC peak areas obtained from the second and third analyses, and the control analysis was reported (eg. $[(0h_{peak\ area} - 11h_{peak\ area})/0h_{peak\ area}]\%$). In general, the percentage difference for a derivative indicated that the peak areas obtained in the subsequent analyses had decreased from that of the control. However, where an increased peak area was observed, a negative percentage was recorded (eg. 1° amines; Table 5.8).

The reproducibility data shown in Table 5.8 were obtained over two days from working drugs solutions of approximately 4 ng/µl. Derivatives of ranitidine, temazepam, haloperidol and terfenadine could not be detected at that concentration. Day 1 data was obtained from replicates which were analysed 10 to 11 h following their derivatisation. During that time, the sample vials were seated in the rack of the autosampler at room temperature. Day 2 data was obtained from replicates which were analysed only 1 to 2 h following their derivatisation.

Consequently, 'within Day 2' data gave the best indication of the reproducibility of derivative formation within a day as Day 1 reproducibility data could not be interpreted independently of stability data. The 'within Day' data was the difference in GC peak areas obtained from replicates (R1 and R2) and reported as a percentage of the smallest replicate peak area $[(R1_{peakarea} - R2_{peakarea})/R2_{peakarea}]\%$, where $R1_{peakarea} > R2_{peakarea}$. The 'b/w Day' data was the difference in mean peak areas obtained from Day 1 and Day 2 replicates and reported as a percentage of the smallest mean peak area.

The data in Table 5.8 indicate that derivatives formed from β-blocking agents and drugs derivatised at 2° amine groups were both stable with time (up to 21 h), and reproducibly formed. As the derivatives were stable for up to 21 h, Day 1 and Day 2 reproducibility data were directly comparable. The data show these PFP derivatives to be reproducible both within the day and from day to day. Of the ß-blocking agents investigated, timolol showed the greatest variation in derivative formation between days and this was likely to be a result of the slightly poorer stability of this derivative over time compared with those of other ß-blocking agents. The instability of this PFP derivative was further indicated by the 'within Day 1' data which showed the percent difference in peak areas between replicates after 10–11 h to be four times greater than that between replicates from Day 2 after 2 h.

Data in Table 5.8 indicate that derivatives formed from drugs derivatised at 1° amine groups were relatively stable with time, but showed greater variation within the group after 16–21 h than did the derivatives of 2° amines. Similarly, the reproducibility data show that PFP derivatives of 1° amines were relatively reproducibly formed both within a day and between days but showed greater variation within the group than was observed with derivatives of 2° amines. The peak areas of most 1° amine derivatives increased after 16–21 h from that at t = 0 h and the increase was dramatic for procainamide.

Data in Table 5.8 indicate that, for drugs derivatised at alcohol groups, individual derivatives differed in their stability with time characteristics. The stability of these derivatives after 16–21 h differed and, for those with poor stability, the deterioration with time was non-reporducible.

Data indicating the reproducibility of formation of individual derivatives at an alcohol group was more consistent within the group of drugs investigated than were the stability with time data. For example, all except hyoscyamine, were relatively reproducibly formed as indicated by the 'within Day 2' data which showed only a small variation in peak areas between replicates for the majority of drugs. Consequently, the large variation in peak areas of derivatives 'between days' reflected derivative instability with time rather than non-reproducible derivative formation.

Table 5.8 lists only three drugs derivatised by PFPA at a phenolic group and these data show derivatives with stability and reproducibility characteristics which were dissimilar within the small test group. Data show that derivatives of oxyphenbutazone and paracetamol were not stable with time and the within day reproducibility of oxyphenbutazone was poor. ('within Day 2' data). Data in Table 5.8 indicate that a peak was not detected for one of the 'within Day 1' replicates for the oxyphenbutazone derivative. Although poor derivative stability may have influenced the presence or absence of a GC peak for this derivative, the large percentage difference between 'within Day 2' replicates indicate that derivative formation was also quite variable. In contrast, the PFP derivative of THC showed both good stability with time and within day reproducibility characteristics.

Table 5.8 show stability and reproducibility data for drug reaction artefacts. These drugs each possessed an alcohol group except disopyramide and carbamazepine which each possessed an amide group. The carbamazepine reaction artefact was derivatised (mono-PFP) while the remaining reaction artefacts were underivatised. Once formed, these reaction artefacts were very stable with time except those of haloperidol and terfenadine. Although no stability data was collected for warfarin, the large percentage difference between the peak areas of 'within Day 1' replicates and 'within Day 2' replicates indicate that the derivative may not have been stable after 10–11 h, and that the deterioration was not reproducible. Consequently, 3 of the 7 reaction artefacts tested in this group were unstable with time. Similarly, the reproducibility data (where obtained) indicate both the reproducible and non-reproducible formation of reaction artefacts in the reaction medium.

The stability and reproducibility characteristics of PFP derivatives formed from two benzodiazepines and from ranitidine were investigated. Data in Table 5.8 clearly indicate that these derivatives were unstable with time.

ii. Linearity.
Table 5.9 shows quantitative chromatographic data for 55 PFP drug derivatives collected from GC analyses over 10 h. Therefore, data from Table 5.9 cannot be interpreted without reference to derivative stability with time (Table 5.8).

Each data point employed in determining linear regression equations was a 'peak area ratio' (Section 2.6.1). Table 5.9 lists both the target *m/z* ion monitored and the reference HC marker for individual PFP derivatives from which the peak area ratios were calculated. Table 5.9 shows linearity data over a defined concentration range for PFP drug derivatives. The concentration range is defined by the 'highest' and 'lowest' drug amounts and 100- to 1000-fold differences in amounts injected were chromatographed for the majority of PFP derivatives (Section 4.3.2.3*ii*). The 'next low' amount refers to the next smallest drug amount derivatised and injected which could not be integrated. Consequently, the limit of detection for a given PFP derivative fell within the range bracketed by the 'lowest' and 'next low' amounts listed in Table 5.9 (Section 5.3.2.4*iii*).

The linear regression equation shown for each derivative described the line of best fit for the $\log_{10}$ of the data points and was fitted to log transformed data points over a concentration range between, and including, the 'lowest' and 'highest' amounts injected as listed in Table 5.9. Further discussion of the linear regression equation for log-transformed data points and its relationship ($y = 10^{C}.x^{M}$) to linear data points is given in Section 3.3.1. The 10^{M} factor is given for PFP derivatives for which a linear regression equation was obtained. The 10^{M} factor is a good indicator of derivative linearity over the concentration range tested (Section 4.3.2.3*ii*).

Linearity data (10^{M}) in Table 5.9 indicate the following.

1. Data for 22% of the PFP derivatives were best fitted by an approximately linear equation (10^{M} from 9.2 to 13.5; r^2 from 0.990 to 0.999). The equation was approximately linear over a 100–2000-fold increase in concentration except for derivatives of oxazepam and oxyphenbutazone where the increase was 20- and 25-fold respectively.
2. No linearity data was obtained for 13% of the PFP derivatives listed as only two data points were obtained in the concentration range investigated (*).
3. Data for 66% of PFP derivatives investigated were best fitted by non-linear equations ($M \neq 1$).
4. Data for 29% of PFP derivatives were best fitted by a curve where the increase in detector response was greater for larger drug amounts injected (10^{M} from 13.8 to 20.4). The data points for the majority of these derivatives covered a 100- to 2000- fold increase in concentration.
5. The data for the PFP derivative of timolol and tranylcypromine were best described by a curve where the increase in detector response was greater for

smaller drug amounts injected (10^M = 6.5 and 5.5 respectively) covering 60- and 100-fold increases in concentration.

6. Data for 14% of the PFP derivatives had 10^M values between 20.4 and 30.
7. Data for 20% of the PFP derivatives had $10^M > 30.0$ and covered an increase in concentration which ranged widely from 60-fold for MDMA to 1200-fold for ephedrine. These data indicate that the untransformed data would be best described by a steeply rising curve with a dramatic increase in detector response being observed following an increase in the amount injected.

Data in Table 5.8 and 5.9 indicate that, where the stability of a derivative with time was poor, the untransformed data points would be best fitted by a power equation ($M \neq 1$). For example, the peak areas of the bis- derivatives of oxycodone and quinine had deteriorated by 81% and 85% respectively after 11 h (Table 5.8). Data in Table 5.9 show that the curve fitted to linear data points for these derivatives would be steeply rising (10^M = 72.4 and 89.1 respectively). Other derivatives shown to be unstable with time in Table 5.8 were found to have similar linearity characteristics to those of oxycodone and quinine (haloperidol, paracetamol, pentobarbitone, quinidine, ranitidine, temazepam, terfenadine). In contrast, however, unstable derivatives of oxazepam and oxyphenbutazone identified in Table 5.8 were relatively linear over the concentration range tested (10^M = 9.5, and 10.5 respectively).

Data in Table 5.9 show that the PFP derivatives of the structurally-related ß-blocking agents and drugs derivatised at a 2° amine function were all relatively linear over a broad range of concentrations although individual drugs varied from this trend (propranolol and paroxetine respectively). Similarly, over a concentration range which increased by greater than 25-fold, drugs which were derivatised at the hydroxyl function were generally best described by power equations (except guaiphenasin, hyoscyamine and THC). The structurally-related drugs all showed similar linearity characteristics (eg phenylethylamine derivatives; ß-blocking agents; reaction artefacts and derivatives of hyoscyamine and hyoscine; derivatives of metoclopramide and procainamide; nortriptyline and protriptyline). The drugs derivatised at a 1° amine group were more variable in their linearity over the concentration range tested than were drugs derivatised at a 2° amine.

Data in Table 5.9 indicate that common linearity characteristics did not generally exist between drugs derivatised at the same functional group.

iii. Detection Limit

The limit of detection for a given PFP derivative fell within the range bracketed by the 'lowest' and 'next low' amounts listed in Table 5.9 (Section 5.3.2.4*ii*). The 'next low' amount was not determined for some derivatives (<x).

Data in Table 5.9 indicate that ß-blocking agents, drugs derivatised at a 2° amine function ($10^M < 20$), and the reaction artefacts of drugs with an amide grouping, were consistently detected at < 100 pg injected (except timolol). Instrument sensitivity was very poor (> 10 ng) for derivatives of the anti-diabetic agents

(sulphonylurea grouping), ranitidine, temazepam and dehydration products haloperidol and terfenadine.

Data in Table 5.9 show similar limits of detection for PFP derivatives of structurally-related drugs (ß-blocking agents; phenylethylamine derivatives except MDMA; the anti-diabetic agents; quinine and quinidine; nortriptyline and protriptyline; metoclopramide and procainamide).

The derivatives with the lowest detection limit were those where the linear regression equation described the broadest concentration range. Therefore, data in Table 5.9 indicate that PFP derivatives with GC peak responses which were approximately linear over the concentration range investigated had detection limits <100 pg injected (except for oxazepam and oxyphenbutazone). Similarly, PFP derivatives where $10^M < 20$ (gently sloping curves), had detection limits < 100 pg injected (except for THC, hyoscine and phenelzine). For PFP derivatives where $10^M > 30$ (steeply rising curves), the detection limit at best was > 200 pg and < 4 ng at worst (except phenobarbitone > 70 pg).

Appendix B.3.1.2 *ii.* and Table B.8 give the limits of detection for some stable PFP derivatives determined following OC and splitless injection. OC injection gave instrument sensitivity greater than, or equal to, that achieved following splitless injection and, in some cases, the increase was significant.

5.4 Discussion

The chromatographic characteristics of a wide range of PFP drug derivatives across different drug groups was investigated following splitless and OC injections. The chromatographic information recorded established a database of observed behaviour for these derivatives on a single chromatographic system. From these data it is possible to estimate and compare the readiness of different drugs to participate in acylation reactions with PFPA, and the apparent 'chromatographic polarity' (Section 3.3.1) of their PFP derivatives.

5.4.1 The Reaction

5.4.1.1 *General*

In the pentafluoropropionylation of drugs listed in Tables 5.7–5.9, the substrate (PFPA), leaving group (C_3F_5O), solvent/catalyst (acetonitrile) and conditions created by the reaction medium were the same for all reactions between PFPA and a drug while the structures of the individual drug nucleophiles were relatively diverse. As an anhydride, the acyl group of PFPA possesses a reactive carbon atom (Appendix C.1). In the presence of acetonitrile both the rates of formation and decomposition of transient tetrahedral intermediates should have been enhanced by nucleophilic catalysis (Appendix C.3). Consequently, the formation or otherwise of PFP derivatives from the different drugs investigated under the given reaction conditions, was heavily dependent on the following:-

1. the readiness of the drug nucleophile to attack the reactive carbonyl carbon atom of PFPA (nucleophilicity) and the effectiveness of the attack;
2. the leaving group properties of the drug nucleophile compared with that of PFPA;
3. steric effects determined by the drug nucleophile which influenced the PFPA/drug interaction; and
4. the relative stability of the PFP derivatives in the reaction medium.

5.4.1.2 *Strength of Drug Nucleophiles*

The general factors which determine the strength of a nucleophile have already been discussed (Section 4.4.2). It should be noted, however, that nucleophiles which react rapidly with a saturated carbon atom in S_N2 substitution reactions may not react rapidly with a carbonyl carbon atom and vice versa. The two sets of nucleophiles obey different rules of reactivity.[5] In general, nucleophiles reacting in acylation reactions must be good proton bases.[5] Consequently, unlike the butylation reaction (S_N2), drugs with carboxylic acid groups (strong acids) do not make good nucleophiles for acylation reactions as the conjugate base is weak.

The nucleophilicity of a given nucleophile participating in an acylation reaction with PFPA is dependent on the electron density at the attacking atom and its capacity to donate electrons to the electron deficient carbonyl carbon of PFPA. With the formation of the transient tetrahedral intermediate, however, the electron density at the reaction centre increases such that the presence of electron withdrawing substituents would ease the nucleophilic addition. Consequently, the attacking atom and its substituents, other functional groups carried by the nucleophile, the strength of inductive and resonance effects around the nucleophilic center, solvation and intramolecular hydrogen bonding, all affect the reactivity of an individual drug nucleophile (Section 4.4.2).

Steric influences also participate in determining the effectiveness of the nucleophilic attack on the acyl carbon, as the larger the bulk of the nucleophile (its size and the space it occupies), the more difficult it is to approach the reactive carbon of PFPA (Appendix C.1). Steric hindrance occurs when contact between the reaction centers of the drug nucleophile and PFPA is made more difficult by interference from groups on the nucleophile that do not otherwise participate.[15]

Consequently, as was found for drugs with diverse chemical structures participating in S_N2 reactions (Chapter 4), a comparison of the relative abilities of structurally-unrelated drug nucleophiles to form tetrahedral intermediates with PFPA would be extremely difficult. The drug nucleophiles involved in the acylation reaction differed both in chemical structure and dimension, and in the attacking atom (N or O). Similarly, some drug nucleophiles contained more than one attacking atom (bis derivatives) which further influenced the rate at which the acylation reaction proceeded.

5.4.1.3 *Leaving Group Properties of Drug Nucleophiles*

To ensure the formation of PFP drug derivatives, the various drug nucleophiles must have poorer leaving group properties than either C_2F_5COOH or $C_2F_5COO^-$

(Appendix C.1 and Figure C.2). As all good leaving groups are weak bases and as all weak bases have strong conjugate acids[5] then the tetrahedral intermediate will decompose into a PFP drug derivative provided the pKa for each drug is greater than that of PFPA.

5.4.1.4 *Resonance Stabilisation of PFPA and PFP Derivatives*
The PFP derivative should experience greater resonance stabilisation than either PFPA or the tetrahedral intermediate. The resonance stabilisation of amides and esters (which result from the acylation of drugs at amine and hydroxyl groups respectively), was greater than that of anhydrides due to their ability to better participate in conjugation with the C=O π bond contributing, therefore, to the stability of the acyl group (Appendix C.1)[5].

Resonance stabilisation is greatest when the participating groups are in the same plane and when this is not the case, the ability to delocalise electrons among the groups rapidly decreases (Section 4.4.2). Due to the diversity in size and structure of the drug nucleophiles investigated, it was possible that through steric hindrance, substituents on some nucleophiles may have forced the participating group out of the plane of the re-established double bond between carbon and oxygen and decreased resonance stabilisation for those derivatives. The resulting derivative may have subsequently decomposed into relatively more stable structures (eg reaction artefacts or other products which were not detected by GC).

5.4.1.5 *The Reaction Medium*
In acylation reactions with acid anhydrides, an acidic (non-aqueous) reaction medium was created which could potentially destroy acid-sensitive PFP derivatives and lead to lower derivative yields (Appendix C.2). Similarly, lower derivative yields have resulted following degradation of derivatives in the presence of excess anhydride.[6,14] For example, dehydration at 1° amide groups to form nitriles has been reported in the presence of excess acid anhydride[6,14] and nitrile formation was observed in the acylation of disopyramide (Table 5.4).

The conditions created by the reaction medium were significant for those drugs which formed unexpected reaction artefacts following their acylation. A dehydrated drug structure was identified for a number of drugs derivatised at an alcohol functional group. In the acylation of oxycodone, enol formation in the reaction medium made available a hydroxyl group for a second derivatisation site. A similar process has been reported for medroxyprogesterone acetate.[6] The bis- derivative observed for desipramine resulted from side-chain dehydrogenation of desipramine in the reaction medium.[13,17] Captopril, H_2-receptor antagonists and the anti-diabetic agents (sulphonylurea grouping) were all observed to form unexpected reaction artefacts within the reaction medium.

Conditions in the reaction medium were also significant for those drugs which formed reaction artefacts following acylation which would commonly be observed as GC artefacts following splitless injection (eg. carbamazepine, hyoscyamine, tolbutamide and chlorpropamide). Clearly, the instability of some underivatised

drugs at elevated injector temperatures was manifested under another set of extreme conditions such as those created in the reaction medium. It is postulated that in the PFPA acylation of untested drugs which are prone to GC artefact formation, that the same GC artefact is likely to be formed as a reaction artefact (derivatised or underivatised) in the reaction medium.

Consequently, in conditions created by the reaction medium, the following was observed.

1. The individual structure of a drug nucleophile made it susceptible to participation in unexpected reactions.
2. The characteristic structure of the drug nucleophile, together with the nature of the PFP-drug bond, determined derivative stability in the presence of excess acid and acid anhydride. As reaction artefacts were not commonly formed from drugs derivatised at a 1° or 2° amine group (except desipramine) but were formed more often from drugs derivatised at an alcohol group, the carbonyl carbon-nitrogen bond appeared to be less reactive than the carbonyl carbon-oxygen bond in the reaction medium.
3. The characteristic structures of underivatised drugs which made them thermally unstable at elevated temperatures were likely to impart instability in the reaction medium.

5.4.1.6 *Drug Nucleophiles*

A reactive drug nucleophile possessed properties which caused it to readily react with PFPA and form a transient tetrahedral intermediate which rapidly ejected C_2F_5COOH or $C_2F_5COO^-$ and resulted in the formation of a PFP derivative of the drug which was stable in the acidic conditions created by the reaction mixture. The reactivity of a drug nucleophile was determined by factors described above (Sections 5.4.1.2–5.4.1.5).

From the data shown in Tables 5.7–5.9, it can be concluded that structurally-related drugs formed nucleophiles with similar reactivities and derivatives with similar properties (stability with heat and time, reproducibility, linearity). For example, the diuretic agents (sulphonamides) were apparently unreactive nucleophiles and no PFP derivatives were produced. Their low reactivity may have resulted from:-

1. electron delocalisation at the nucleophilic center (low nucleophilicity),
2. the sulphonamide being either greatly sterically hindered or the better leaving group.

Alternatively, the sulphonamides may have reacted with PFPA, but the product of acylation:-

1. possessed less resonance stability than PFPA,
2. was unstable in the acidic conditions created by the reaction mixture, or
3. was thermally labile and completely decomposed during GC analysis (Section 5.4.2).

In contrast, ß-blocking agents were reactive nucleophiles under the present reaction conditions which allowed them to approach PFPA readily and form the tetrahedral

intermediate. The ß-blocking agents possess hydroxyl and amine groups which have poorer leaving group properties than either C_2F_5COOH or $C_2F_5COO^-$. The bis-PFP derivatives formed had greater resonance stabilisation (esters and amides) than PFPA and were stable in the reaction medium and during GC analysis.

Table 5.7 listed the derivatives formed from drugs with different functional groups. Where bis- derivatives were formed there was more than one reactive nucleophile present in the drug structure (eg. ß-blocking agents, guaiphenasin, desipramine). The formation of a single major PFP-drug product indicated that the drug nucleophile had sufficient reactivity to progress through the successive steps of the acylation reaction and form a derivative that was stable both in the reaction medium and under the conditions required for chromatographic analysis. As might be expected, reactive drug nucleophiles were generally those with attacking N atoms (amines) or attacking O atoms (hydroxyl). As exceptions were observed, however, the individual structure of the drug nucleophile together with the functional group derivatised was significant in determining reactivity. For example, some drugs which possessed amine and hydroxyl groups were unreactive nucleophiles and no derivative was formed due either to low nucleophilicity or steric hindrance (eg. prazosin, chloroquine, theophylline, benzhexol). Other drugs with these functional groups (eg chlordiazepoxide, fluphenazine, prednisolone, procyclidine) made more reactive nucleophiles as PFP-related products were chromatographed. The multiple products formed by these drugs, however, indicated a high degree of derivative instability. The unstable derivatives were short-lived and decomposed/rearranged into related products either within the reaction medium or during chromatographic analyses. Similarly, the formation of reaction artefacts from drugs with hydroxyl functions suggested that either the drug or the PFP derivative of the drug was unstable in the reaction medium. For these drugs, a greater degree of stability was attained following their rearrangement/dehydration than through the simple formation of a PFP derivative from the drug.

Clearly, reactivity of drug nucleophiles were determined by their individual structures.

5.4.1.7 *Catalysis*

Acylating reagents can be used either alone, or in combination with a variety of different catalysts. In the literature, strong bases affect the formation and decomposition of the tetrahedral intermediate leading to shorter reaction times, lower reaction temperatures, greater derivative yields and derivatisation of a variety of different functional groups.[1,2,7-12] In the present work, however, the base-catalysed acylation of amphetamine, methylamphetamine, morphine and codeine by the strong bases (pyridine, DEA) proved detrimental to derivative formation. These catalysts were also difficult to remove following reaction and generated 'dirtier' chromatograms with additional peaks that interfered with the peaks of interest. The yellow reaction mixture observed during the derivatisation of morphine and codeine in the presence of either catalyst has been observed with pyridine and reported to contaminate the electron capture detector.[6] Similarly, the residue formed by the

side-reaction of pyridine with acid anhydrides (Appendix C.3) was also produced with PFPA although it was soluble in the GC injection solvent.

Had the test drug group contained a greater number of drugs with different functional groups it is possible that catalysis may have been apparent with these strong bases. The improvement in derivative yield, however, would have had to be very significant to offset the practical problems presented by these catalysts. With the use of acetonitrile as a catalyst, the appearance of the reaction mixture and chromatograms were similar to those produced by PFPA and ethyl acetate. Acetonitrile appeared to be behaving simply as a solvent, although the yield of mono-PFP codeine was increased.

The catalysis of drug acylation with acetonitrile would, in theory, proceed as it did for pyridine (Appendix C; Figure C.1; Equation 2). A protonated acetonitrile-acyl compound would result from the reaction of PFPA and acetonitrile following the rapid ejection of the carboxylate ion (the weakest base and better leaving group) from the unstable tetrahedral intermediate. The protonated compound produces a greater positive charge on its carbonyl carbon, making it more attractive to attack by a drug nucleophile. Acetonitrile could subsequently accept acids formed during the acylation. However, in comparison to pyridine, acetonitrile is only a very weak base despite the lone electron pair of the nitrogen atom. Although the nitrile group is shown as a triple bond, the greater electronegativity of nitrogen results in considerable polarisation of the triple bond such that there is a partial positive charge on the carbon and a partial negative charge on the nitrogen.[4] The electronegativity of nitrogen in this unsaturated state causes its electron donor capacity to be diminished from that in saturated states (amines).[4] It is likely then, that amine (and hydroxyl) drug nucleophiles would have better nucleophilic properties (greater electron donor capacity) than acetonitrile and more readily attack the carbonyl carbon of PFPA. Similarly, nitriles can be protonated only under very strongly acidic conditions ($RCC{\equiv}N^{+}H$) and is only partially protonated in sulphuric acid solution.[5] Although acylation with PFPA does proceed in an acidic medium, acetonitrile may be only a weak proton acceptor. Acetonitrile must be a very weak basic catalyst and although it has been said to catalyse the reactions of both amine and hydroxyl groups with acid anhydrides[1], it may simply be a good solvent for these acylation reactions.

5.4.1.8 *Reaction Time*

The rate of formation and decomposition of the tetrahedral intermediate determines the rate of PFPA derivatisation of drugs (Appendix C.1). Data in Table 5.3 showed that PFP derivative formation occurred within 5 min for some drugs (desipramine, codeine) while up to 120 min was required for others (amphetamine, terfenadine) despite derivatisation at similar functional groups (1° amine, alcohol). Clearly, the individual structures of these drug nucleophiles were determining the rates of formation of the intermediate in addition to the functional group derivatised. Consequently, formation was slower for amphetamine and terfenadine than for desipramine and codeine due to differences in the reactivity of the individual nucleophiles as determined by structure. The individual structural characteristics of a particular drug nucleophile governed its nucleophilicity, the ease with which the

nucleophile approached the carbonyl carbon (steric hindrance) and the electron density at the reaction center. For a given drug nucleophile, each of these factors influenced the rate of formation of the tetrahedral intermediate differently, despite similarities in the functional group derivatised.

5.4.2 PFP Derivatives

5.4.2.1 *Overview*

Approximately 90 drugs with functional groups capable of being acylated were derivatised with PFPA and their derivatisation products (if formed) were chromatographed and detected by low resolution EI mass spectrometry. Data in Table 5.7 identified derivatisation products and listed their general chromatographic and mass spectral characteristics. It was found, however, that the low resolution EI mass spectra obtained from these products were not easily interpreted in terms of the structure of the underivatised drug and the predicted derivative. For 22 of the drugs investigated, additional mass spectral data (CI and accurate mass determination) were necessary to identify the derivatisation products and sites of derivatisation (Table 5.4). It was found that attempting either to predict the derivative formed from some drugs, or to identify some new PFP derivatives from their low resolution EI mass spectra, was unsuccessful for the following reasons:-

1. the absence of a molecular ion,
2. the potential for unusual mass spectral fragment rearrangements, and
3. the potential formation of reaction artefacts (dehydration of the drug or the formation of unique derivatives involving chemical reactions which could not be predicted).

Similarly, it has been reported in the literature that a number of compounds other than drugs are known to form derivatives other than those expected.[1,6] It was shown that during the derivatisation of drugs with PFPA, several side-reactions can potentially occur within the reaction medium to produce unusual derivatisation products which may or may not be subsequently acylated. Consequently, the identity of the derivative produced following the reaction of a new drug with PFPA should not be assumed, particularly if the drug possesses alcohol or 1° amide groups. From the data summarised in Table 5.7 for the drugs investigated, some general statements about the reaction between PFPA and drugs can be made.

1. Mono- and bis- PFP derivatives are generally formed from drugs derivatised at 1° and 2° amine and hydroxyl groups. Some drugs which possess these functional groups, however, form multiple unidentified products.
2. Drugs with sulphonamide and sulphonylurea groupings, either form no derivatives, or derivatives sensitive to elevated temperatures.
3. Similar reaction artefacts and/or derivatives are formed from structurally-related drugs (structural analogues) and behave similarly when chromatographed.
4. Reaction artefacts are not commonly formed from drugs derivatised at their 1° and 2° amine functions.
5. Reaction artefacts commonly occur following derivatisation of drugs at their alcohol functional group (often the dehydrated drug), sulphonylurea or 1° amide group.

6. GC artefacts which would commonly be chromatographed following splitless injection are formed and sometimes derivatised within the PFPA reaction mixture.

No derivatives were formed from the structurally-related diuretic drugs (sulphonamide grouping) and it was not the proximity of the SO_2 group to the NH_2 group (site of derivatisation) which prevented their derivatisation, as derivatised reaction artefacts were formed from the anti-diabetic drugs (chlorpropamide and tolbutamide). A PFP derivative may not have been detected for several reasons as follows.

1. The diuretic drugs are much larger, bulkier compounds than the anti-diabetic drugs and steric hindrance may have prevented the formation of the tetrahedral intermediate.
2. A PFP derivative may have been formed which was too unstable to persist either in the reaction medium or under chromatographic conditions. The derivatised reaction artefacts of the anti-diabetic agents provided evidence of the instability of compounds derivatised at a sulphonamide group as they were unstable at injector temperatures.
3. A derivative and/or degradation products were formed which did not chromatograph.

The sulphonamide grouping has been successfully derivatised with trifluoroacetic and heptafluorobutyric anhydride in the presence of TMA.[18] However, smaller, less bulky compounds than the diuretic drugs were derivatised which were very similar to the reaction artefacts of the anti-diabetic agents (eg. benzenesulphonamide and *n*-ethyl- and *n*-phenyl-benzenesulphonamide). Interestingly, the perfluoroacyl derivatives of benzenesulphonamide could not be chromatographed.[18]

It should be noted that the factors above which may have prevented detection of diuretic drug derivatives may also explain why no derivatives were detected for other drugs with functional groups capable of being acylated. Alternatively, the use of appropriate catalysts and optimisation of chromatographic conditions may improve derivative formation and detection. For example, no chloroquine derivative was detected under the present conditions but mono-PFP chloroquine was formed in the presence of TMA and, following on-column injection, was chromatographed on a 25 m OV-1 fused silica capillary column (0.33 mm i.d; oven temperature 125°C to a maximum temperature of 220°C).[19] The analytical conditions ensured derivative formation through nucleophilic catalysis and the chromatographic conditions selected ensured that the derivative was exposed to low oven temperatures for a short period of time (retention time of 7 min) during its chromatography.

The ß-blocking agents were the largest group of structurally-related drugs investigated and the influence of subtle structural changes on the formation of PFP derivatives can be demonstrated for these drugs. Predictable bis-PFP derivatives of each drug except labetalol and sotalol were formed. Labetalol formed an unexpected derivative identified as the bis-PFP derivative of the doubly dehydrated drug (Table 5.4). Although dehydration of labetalol was not predicted, some difference in the

PFPA–drug reaction might have been expected given the structural difference of the side-chains between labetalol and other ß-blocking agents (Figure 5.3). The identity of the sotalol derivative was not immediately apparent from the low resolution EI mass spectrum. Having determined that dehydration reactions could potentially occur in the reaction medium, however, and given the structural similarity between labetalol and sotalol at their side-chains, it was not unexpected that the derivatisation product of sotalol was identified as the mono-PFP derivative of the dehydrated drug (confirmed by low resolution EI mass spectrometry).

In general, the ß-blocking agents formed a single bis- derivatives, but multiple derivatives were produced following the derivatisation of pindolol and timolol. Although the side-chain at which derivatisation occurred was similar for these drugs, there were substantial differences from the other drugs in the structures of the core of these molecules (Figure 5.3) which may have affected the stability of derivatives (Section 5.4.1.6).

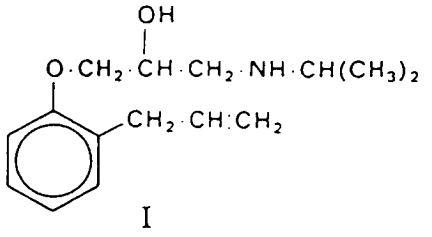
The structural similarities of the sidechains at which derivatisation occurred for the ß-blocking agents predictably resulted in similarities in their low resolution EI full-scan mass spectra, and common fragment ions (*m/z* 366 and *m/z* 408) were formed. Similarly, of the phenylethylamine derivatives with a 2° amine function, a common fragment ion *m/z* 204 was formed.

Practical data was generated with regard to the stability of some PFP derivatives at splitless injection and oven temperatures (Tables 5.5–5.7), the reproducibility of derivative formation and their stability with time (Table 5.8), the linearity of derivatives over wide concentration ranges and instrument sensitivity to PFP derivatives (Table 5.9).

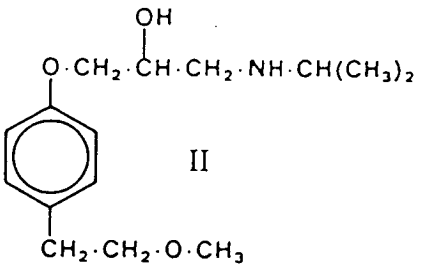
5.4.2.2 *Stability at Elevated Temperatures*

The chromatographic stability of some PFP derivatives was initially investigated using three different injection techniques and was found to improve when exposure to elevated injector temperatures was removed (Section 5.3.2.2). The EPC injection was a splitless injection. The greater gas flow through the injector at 40 psi, however, minimised losses of high boilers due to injector discrimination and speeded the transfer of the sample from the hot injector to the front of the column (Appendix B.3.1). Although sample exposure to the injector temperature following injection at 40 psi was reduced from that following injection at 10 psi, all sample components still experienced 260°C for some period of time (seconds). The instability of some derivatives to elevated injector temperatures was illustrated with the chromatography of the etafedrine derivative. This derivative degraded into several unresolved peaks (GC artefacts) following EPC and splitless injections while following either SPI and OC injections the integrity of its structure was maintained.

The SPI injection was also a splitless injection, but under SPI conditions individual sample components were exposed to the minimum temperature required to volatilise them rather than to a constant 260°C. The SPI injector ramped rapidly (180°C/min) to 320°C which implies that, although a sample component was volatilised at its



I



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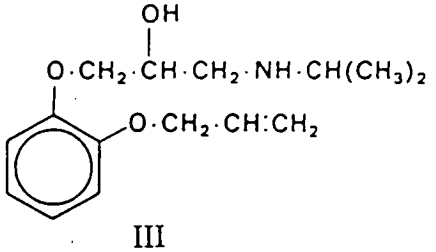

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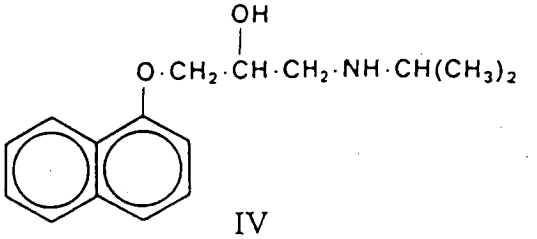

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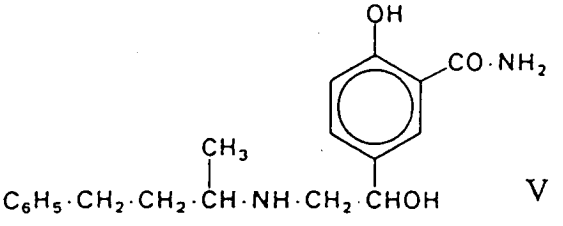

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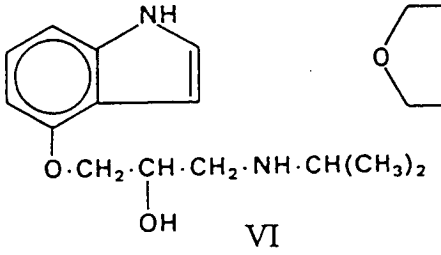

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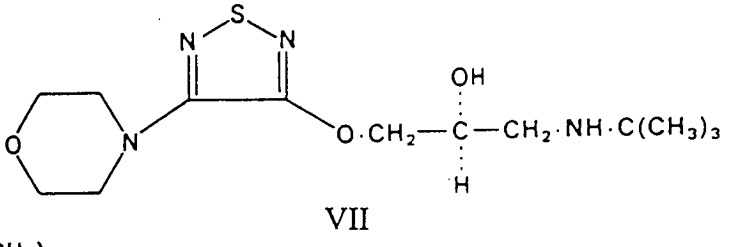

VII

Figure 5.3: The structures of ß-blocking agents - alprenolol (I), metoprolol (II), oxprenolol (III), propranolol (IV), labetalol (V), sotalol (VI), pindolol (VII) and timolol (VIII).[16]

minimum temperature, it was exposed to higher temperatures during the time required to flush it onto the front of the column. Mono-PFP hyoscyamine was only chromatographed following OC injection indicating that the brief exposure to excessive injector temperatures it experienced during SPI injection caused its degradation.

Clearly both EPC and SPI injection could result in the degradation of thermally sensitive derivatives and/or the formation of GC artefacts, but these occurrences would be very greatly reduced following SPI injection. Under OC injection conditions, the sample was transferred as a liquid directly onto the front of the column without exposure to heat. Therefore, following OC injection the peak areas of thermally unstable derivatives would be greatest and the peaks of GC artefacts absent. Consequently, the data in Table 5.5 indicated that SPI injection tended to mirror either EPC (splitless) injection or OC injection as this injection technique had some of the characteristics of both splitless and OC injection.

Data in Section 5.3.2.2 indicate that the chromatography of temperature-sensitive derivatives was not consistent among the three splitless injections. Often a small peak was detected following splitless injection on the Kratos Concept ISQ system (EPC) which was absent following splitless injections on the other two systems. This resulted from the difference in gas flows through the three systems. The gas flow on the EPC system was higher (15 psi 1 min after injection) than the other systems (10 psi) causing sample components to elute more rapidly and experience lower oven temperatures during elution. The detection of these small peaks following splitless injection on the EPC system indicated the presence of thermally-sensitive derivatives which were not completely destroyed in the injector. As chromatography on either of the other two resulted in sample components eluting more slowly at higher oven temperatures, these same derivatives were not detected as they degraded on the column during elution.

There was an anomaly in data from Table 5.5 where the mono-PFP timolol was absent following SPI injection but chromatographed following both EPC and OC injection. This can have resulted only from the non-reproducible formation between days for that derivative. Although the reproducibility of this minor timolol derivative was not investigated, the reproducibility and stability of the major derivatisation product (bis- derivative) did show greater variability between replicates than any of the other ß-blocking agents investigated (Table 5.8).

The increase in intramolecular energy at injector temperatures for thermally-sensitive derivatives was such that bonds were ruptured/reformed and the derivative fell apart in the injector. Degradation products and/or structurally rearranged GC artefacts were formed. The data in Table 5.6 indicate that degradation products/GC artefacts were detected for some derivatives while no GC peaks were apparent for others. Although the degradation products were flushed onto the front of the column with the carrier gas, only those able to be chromatographed were detected.

Although conditions created by elevated injector temperatures were removed

following OC injection, the derivatives were still subject to rising oven temperatures. For derivatives with short retention times, the exposure was short and the oven temperatures relatively low. The longer the retention time of the derivative, however, the greater was its exposure to higher oven temperatures up to a maximum of 300°C. Derivatives unstable at oven temperatures also formed degradation products and artefacts and, unless substantial and sudden degradation occurred once a critical oven temperature was achieved, these products were not focussed on the column and were chromatographed as broad irregular complex peaks if able to be chromatographed at all. The tailing peaks chromatographed for the unstable derivatives would have had reduced peak areas as a result of their degradation on the column. If rapid degradation occurred at a critical oven temperature, then the unstable derivative may be completely destroyed and only degradation artefacts remain. Consequently, for the derivatives which were described with 'T' and 'R' chromatographic characteristics (OC injection; Table 7.6), the unresolved GC peaks were a complicated mixture of unstable derivatives and their degradation products which may themselves be thermally unstable. Clearly, the identification of derivative/s formed in the reaction mixture from their corresponding artefacts would be very difficult. The splitless injection of these temperature-sensitive derivatives, however, resulted in the instantaneous formation in the injector of the some of the same degradation products which, following OC injection, were formed on the column during elution. Following splitless injection, the original derivative/s were completely destroyed.

It is interesting to note that, for the majority of the drugs listed in Table 5.7, RI values were < 2500. Reaction artefacts of haloperidol and terfenadine were the only late eluting derivatives. It was shown in Table 5.5 that the peak area of dehydrated haloperidol was reduced following splitless injection. It was also observed that longer elution times (higher temperatures during chromatography) were detrimental. Consequently, it may be that both artefacts of haloperidol and terfenadine suffered degradation during their chromatography reflected by reduced peak areas. Similarly, for drugs for which no derivative was detected, it may be that derivatives with RI values > 2500 were formed in the reaction mixture which were completely degraded during their chromatography such that it appeared that no derivative had been formed.

From data in Tables 5.5–5.7 it was found that a greater number of PFP derivatives was successfully chromatographed following OC than splitless injection. Data in Table 5.7 indicated that PFP-related compounds showed differing degrees of instability with increased temperatures ranging from unstable at oven temperatures to stable at both oven and injector temperatures. In general, drugs derivatised at their amine and hydroxyl groupings showed the greater stability during chromatography, although exceptions were observed. Consequently, the individual structures of drugs were critical in determining the thermal stability of their PFP derivatives.

5.4.2.3 *Stability with Time and Reproducibility*

Table 5.8 showed stability with time data for some PFP derivatives. These data were a measure of the relative rates of PFP derivative decomposition with time into other

product/s within the injection solvent. The decomposition products would only be detected if they were able to be chromatographed (unchanged), but no attempt was made to detect or identify decomposition products.

It was preferable to remove the excess PFPA prior to injection as irreversible alteration of the GC column, corrosion or other damage to the GC-MSD system may have resulted from its chemical reactivity.[1] In removing the reaction medium, however, the stability of some PFP derivatives with time may have been compromised. That is, some derivatives may have been stabilised by conditions in the reaction medium which were changed when excess PFPA and pentafluoropropionic acid were removed. For example, by virtue of double bond migration allowing keto-enol tautomerism to produce a hydroxyl group, the bis-derivative of oxycodone was formed in the reaction medium. Ketone formation may have been preferred in the injection solvent, however, which led to the rapid deterioration of bis-PFP oxycodone with time (Table 5.8). Similarly, the reaction artefacts of haloperidol and terfenadine may have been stabilised by conditions in the reaction medium but rapidly decomposed in the injection solvent.

Table 5.8 showed reproducibility data for the formation of derivatives on a given day and between days for some drugs. The reproducibility of derivative formation would be a measure of what proportion of the drug consistently formed a tetrahedral intermediate and expelled the leaving group to form the PFP derivative, and what proportion of the derivative was consistently stable in the reaction medium and under chromatographic analysis. Where reaction artefacts were formed, the reproducibility data would also be a measure of the proportion of PFP drug derivative consistently converted to that artefact in the reaction medium.

The proportions measured would, therefore, provide a derivative yield (x% of the starting drug) and the reproducibility of that same yield being formed following another preparation ($\pm y$%). No reference standards for PFP derivatives were synthesised and, therefore, no measure of derivative yield was made. With the derivatisation of replicate drug solutions, however, the reproducibility of formation of a given unknown yield of derivative/reaction artefact could be obtained by comparing the peak areas chromatographed for that derivative in the replicates. Provided the derivative/reaction artefact was stable with time, then data in Table 5.8 showed that where the variation between the peak areas was small, a similar amount of drug was converted to a PFP derivative/reaction artefact. Where broad variations were recorded between replicates, similar amounts of drug were not converted to PFP derivatives as a result of one or more non-reproducible steps in the acylation reaction.

Where the between day variation was large for replicates which on a given day had shown small variation, different but reproducible derivative yields had been produced on each day (provided the derivative was stable with time). The between day variation in derivative yield was due to subtle changes in either the reaction medium, or chromatographic conditions between days. Either circumstance might have caused more or less drug to be converted to its PFP derivative, to remain stable in the

reaction medium, or to be chromatographed.

The stability data in Table 5.8 indicate, however, that not all derivatives were stable with time. Under this circumstance the 'within Day 2' data in Table 5.8 best describes the reproducibility of derivative formation on a given day while 'within Day 1' data provides supporting evidence of derivative instability. Therefore, depending on the stability of derivatives with time, the 'b/w Days' data sometimes reflected derivative instability and at other times reflected differences in derivative yield.

It was indicated in Section 4.4.2.3 that exhaustive studies regarding the stability and reproducibility of derivative formation for each of the target drugs were not warranted or possible within the time span of the project. Rather, it was necessary to determine whether acylation of a drug was promising enough for subsequent extraction experiments with target drugs (Chapter 7). It is also possible that further experimentation would have revealed that results recorded for the replicates (reproducibility data) and the decomposition of a single solution (stability data) represented 'out-lying' samples.

Although data in Table 5.8 represents only two pairs of replicates and the decomposition of a single solution, some general trends did emerged as follows.

1. In general, PFP derivatives that were very stable with time were also reproducibly formed, regardless of the functional group derivatised.
2. The PFP derivatives of ß-blocking agents and drugs derivatised at their 1° and 2° amine groups were formed reproducibly (within and between days) and, once formed, were stable after 16 - 21 h.
3. Drugs derivatised at a 2° amine group showed greater consistency in the stability and reproducibility characteristics of their derivatives within the group than did those derivatised at a 1° amine group.
4. The peak areas of some drugs derivatised at an amine function (particularly at 1° amines) increased at 11 and 16–21 h from those recorded at t = 0.
5. The PFP derivatives of drugs derivatised at alcohol functional groups were formed relatively reproducibly and were relatively stable with time except those of oxycodone and quinine and its stereoisomer
6. The PFP derivatives of the small test group of drugs derivatised at phenolic functional groups were not similar in their stability and reproducibility characteristics.
7. The stability of reaction artefacts formed from drugs possessing an alcohol function was inconsistent within the test group. Where data were available for these drugs, most reaction artefacts were relatively reproducibly formed. Reaction artefacts formed from drugs with a 1° amide group were both stable with time and reproducibly formed.
8. The derivatives of the benzodiazepines tested were neither stable with time nor reproducibly formed.

The variation between replicates recorded for some derivatives may reflect problems in the chromatography of those derivatives rather than their instability or non-

reproducibility. In general, the esters and amides formed following acylation have better chromatographic properties than the polar underivatised drugs. However, some amides retain a residual polar character and have a tendency to tail unless chromatographed on a moderately polar stationary phase.[1] The residual polar character may also cause them to adsorb to active sites on the column. Consequently, the influence of chromatographic factors on quantitative data may be greater for drugs derivatised at amine functions than at hydroxyl functions.

The data in Table 5.8 tend to support this. The data in Table 5.8 gives the percent difference in derivative GC peak area at 11 and 16-21 h from that at t=0 (100%). It would be expected that the GC peak area would either remain constant, or decrease, with time. The peak areas of derivatives formed from amines, however, increased with time (peak areas > 100%), particularly those formed from 1° amines and the peak area was greatest following the third analysis (eg. methylphenidate, procainamide). This implies that either the derivative continued to form in the absence of PFPA in the injection solvent as time elapsed (which is impossible) or problems in chromatography affected the peak areas of derivatives via altered peak shape, errors in integration and adsorption to the column (Section 3.4). The data in Table 5.8 suggest that adsorption to active sites on the column was influencing the stability data for PFP derivatives which formed amides.

The stability, reproducibility and linearity data were collected from a sequence of 17 consecutive analyses. The control sample (t = 0) was the first sample in the sequence followed by samples at different drug concentrations for linearity and the Day 1 replicates. The 11 h and 16–21 h stability samples were samples 14 and 17 respectively. Derivative adsorption to the column would be reduced for samples analysed later in the sequence as active sites were filled with adsorbed sample from previous GC analyses. Clearly, the problem of adsorption was most serious for derivatives formed at 1° amines although, as it was observed for other amide derivatives, the individual structures of drugs also contributed to the polarity of the amide grouping. For example, although derivatives of metoclopramide and procainamide were structurally similar, their polarities must have been quite different as the derivative of procainamide was much more prone to column adsorption than that of metoclopramide.

The greater variation in 'b/w Days' data observed for 1° amines compared with 2° amines probably reflected the decreased adsorption of these derivatives for Day 2 replicates. Therefore, in quantitative analyses of derivatives formed at amine groupings, particularly at 1° amines, prior column priming would be advisable.

Data in Table 5.8 indicates that stability and reproducibility data are not similar for drugs derivatised at a phenol function. Given that the individual structures of drugs strongly influence all aspects of derivative formation and their properties, the lack of consistency in reproducibility and stability data for these derivatives is not surprising as they are structurally very different compounds.

5.4.2.4 *Linearity*

Table 5.9 showed linearity data for 55 PFP derivatives. No data was obtained for 13% of derivatives. For the remaining derivatives, data indicated that linear to gently sloping curves best described the concentration range tested for approximately 50% of the derivatives investigated ($10^M < 20$). Common linearity characteristics did not generally exist between drugs derivatised at the same functional group. For example, drugs derivatised at the 2° amine function were relatively linear over a broad range of concentrations, but variations did occur (eg paroxetine). Similarly, over a concentration range which increased by > 25 fold (broad concentration range), drugs derivatised at the hydroxyl function were generally best described by power equations. In contrast, structurally-related drugs had similar linearity characteristics (eg ß-blocking agents).

Data in Table 5.9 were collected over 10 h. The data points obtained for stable PFP derivatives would not be affected by the time elapsed between derivatisation and analysis. As the time between derivatisation and analysis increased for PFP derivatives with relatively poor stability, the peak area obtained for a given data point would be proportionately smaller than if it had been analysed immediately following its derivatisation (Table 5.8). As the drug amounts which would define the upper and lower extremes of the concentration range were analysed earliest (Section 2.6.1), a curve rather than a straight line might be expected for derivative which was unstable with time. The data in Tables 5.8 and 5.9 confirm this as, where the stability of a derivative with time was poor, the linear data points were found to best fitted by power equations (Section 5.3.2.4 *ii.*). In contrast, unstable derivatives of oxazepam and oxyphenbutazone were relatively linear over the concentration range tested. These derivatives, however, gave responses over much narrower concentration ranges than other derivatives found to give linear GC responses.

A curve rather than a straight line would best describe data over the concentration range tested where the chromatography of the derivative was affected by column adsorption. The effect of column adsorption on stability data shown in Table 5.8 was discussed in Section 5.4.2.3. Similarly, as linearity data was generated, derivative adsorption to the column was reduced for samples analysed later in the sequence as active sites were filled with adsorbed sample from earlier analyses. Consequently, where column adsorption was significant, power rather than linear equations described the concentration range tested.

It would not be unreasonable to assume that, as the residual polar character of amides was governed by overall drug structure, the polarity or otherwise of esters may be influenced by their drug structure. Data in Table 5.9 indicate this to be the case. It is postulated that as power equations described the broad concentration ranges of many derivatives formed at hydroxyl groups, the residual polarity of these derivatives resulted in column adsorption. The column adsorption influenced the absolute peak areas eluted and had the greatest impact for the smallest amounts injected (smallest absolute peak areas). In contrast, derivatives of guaiphenasin, hyoscyamine and THC were chromatographed without column adsorption due to their relatively non-polar characteristics.

Both linearity over a broad concentration range (Table 5.9) and good peak shapes for 50% of PFP drug derivatives indicate that through pentafluoropropionylation, the polar functional groups of these drugs were successfully eliminated, preventing their undesirable interaction with active sites on the column and excessive interaction with the stationary phase. The residual polarity apparent for the remaining 50% of derivatives investigated limited the concentration range over which they were linear. Through acylation, the chromatographic response for the majority of these polar drugs has been either significantly improved or maximised from that observed following their chromatography as underivatised drugs (Tables 3.2 and 3.4).

5.4.2.5 *Detection Limit*

Data in Table 5.9 indicate that similar detection limits did not generally occur between drugs derivatised at the same functional group while those of structurally-related derivatives were similar. For relatively polar derivatives susceptible to column adsorption, linearity data from Table 5.9 suggests that the GC peaks chromatographed following the lowest amounts injected were so affected by losses to active sites on the column that they were significantly reduced or disappeared. All drug amounts injected which appeared in the 'next low' column (except <x) were unable to be integrated as a result of either complete or partial column adsorption at that level. Therefore, those derivatives with chromatographic linearity described by power equations were also those for which detection limits were relatively higher. The same outcome was observed for polar and non-polar underivatised drugs (Table B.12).

The detection limit gave an indication of the yield of derivative from the reaction of PFPA and drug. Where a peak was detected following the derivatisation of a 20 pg amount of drug, then the yield of derivative from the reaction must have been high as the detection limit of the MSD is at pg levels. In contrast, where a peak could not be detected following the derivatisation of a 4 ng amount of drug, then it can be concluded that the yield of derivative from the reaction was poor. The determination of whether or not the derivative yield from a particular PFPA-drug reaction was high, however, is complicated by the affects of column adsorption, derivative instability with time and excessive temperatures, and the reproducibility of derivative formation. A linear regression equation was not obtained for derivatives of haloperidol, ranitidine, temazepam and terfenadine. Only the two largest amounts which were injected of these drugs produced a GC peak which could be integrated. Data in Table 5.8 indicated that these derivatisation products were very unstable with time. No reproducibility data was obtained as these derivatives were not detected at the level injected (ng quantities) even though the 'within Day 2' replicates were analysed almost immediately. Therefore, derivatives of these drugs were not only unstable but produced a very low yield of product.

References

1. "Handbook of Derivatives for Chromatography."
Blau K, King G (Eds); 1978, Heydon & Sons Ltd. London, 104 - 145

2. "Derivatisation reaction in the gas-liquid chromatographic analysis of drugs in biological fluids."
Hulshoff A, Lingeman H; J Pharm & Biomed. Anal. (1984) 2, 337 - 380

3. "Derivatisation for gas and liquid chromatography.
Ahuja S; Chemical Analysis: A series of Monographs on Analytical Chemistry and its Applications.(1986) 85, 19-90

4. "Properties and reaction of bonds in organic molecules."
Reid K F; 1968, 373 - 394; Longmans Green & Co Ltd. United Kingdom.

5. "Organic Chemistry."
Kemp D S, Vellaccio F; 1980, Worth Publishers Inc. USA; 351 - 382

6. "Derivative formation in the quantitative gas-chromatographic analysis of pharmaceuticals. A review"
Nicholson J D; The Analyst (1978) 103, 1-28

7. "N-Methylimidazole as a catalyst for analytical acetylations of hydroxyl compounds."
Connors K, Pandit N; Anal Chem. (1978) 50, 1542 - 1545

8. "Rapid acetylation of a dihydroxy compound by 4-(dimethylamino)-pyridine catalysis."
Rowe E, Machkovech S; J Pharm. Sci. (1977) 66, 273 - 275

9. "The pyridine-catalysed acylation of phenols by carboxylic acid anhydrides in carbon tetrachloride."
Bonner T, Hillier K; J.C.S. Perkin II (1973) 1828 - 1833

10. "Quantitative gas chromatographic determination of picogram quantities of amino and alcoholic compounds by electron capture detection."
Walle T, Ehrsson H; Acta Pharm. Sueccica (1970) 7, 389 - 406

11. "Diperfluoroacylation of primary amines."
Ehrsson J, Brötell H; Acta Pharm. Sueccica (1971) 8, 591 - 597

12. "Quantitative gas chromatographic determination of picogram quantities of phenols."
Ehrsson J, Walle T, Brötell H; Acta Pharm. Sueccica (1971) 8, 319 - 328

13. "Synthesis and structure of an enamine bis(trifluoroacetyl) derivative of desipramine."
Walle, T, Ehrsson H, Bogentoft C; Acta Pharm. Suecica (1972) 6, 509-513

14. "Degradation of perfluoroacyl derivatives of tocainamide and some of its analogues in the presence of an excess of anhydride reagent.
Gyllenhaal O, Hoffman K; J Chromatogr (1986) 355, 127 - 140

15. "Mechanism and structure in organic chemistry."
Gould E S; 1959, 250 - 280; Holt, Rhinhart and Winston Inc. USA.

16. "Clarke's Isolation and Identification of Drugs."
Moffat A (Ed.); 1986, 2nd Edition, The Pharamceutical Press, London

17. "Quantitative gas chromatographic determination of nanogram levels of desipramine in serum."
Ervik M, Walle, T, Ehrsson H; Acta Pharm. Suecica (1970) 7, 625 -634

18. "Determination of sulphonamides by electron-capture gas chromatography."
Gyllenhaal O, Ehrsson H; J Chromatogr. (1975) 107, 327-333

19. "Determination of chloroquine and its major metabolites in blood using perfluoroacylation followed by fused-silica capillary gas chromatography with nitrogen-sensitive detection."
Churchill F, Mount D, Schwartz I; J Chromatogr. (1983) 274, 111-120

Chapter 6.

EXCLUSION OF SOME TARGET DRUGS FROM FURTHER INVESTIGATION

6.1 Introduction

The chromatographic information obtained for drugs and drug derivatives in Chapters 3–5 enabled a database to be established which recorded the observed behaviour of these analytes on a non-polar chromatographic system. From these data it was possible to estimate and compare the apparent 'chromatographic polarity' (Section 3.3.1) of individual drugs. Comparisons were made between the chromatographic properties of underivatised and derivatised drugs and their structural characteristics which permitted the formation of either butyl or PFP derivatives (Sections 3.4, 4.4 and 5.4). In many ways, these data add to the published information which is fragmentary as it deals mainly with either selected drugs, or single drug groups, and does not permit comparison across groups. The drugs studied were representative of a wide range of chemical structures and functionalities. Consequently, the chromatographic properties of other drugs and their suitability to participate in derivatisation reactions with either *n*-butyl iodide or PFPA may be reliably estimated provided they are structurally similar to a drug already investigated.

In Section 3.1 it was stated that the development of a comprehensive drug extraction strategy could not be explored without first being confident that target drugs could be chromatographed and detected at concentrations equivalent to expected therapeutic blood concentrations. The primary aim of experiments in Chapters 3–5 was to generate data which would define the limitations of the GC/MS as an analytical tool for a large group of structurally-unrelated drugs which possess a variety of different functional groups.

It was observed that manipulation of some chromatographic variables (eg. column phase ratio; absolute gas flow through the column; exposure to elevated temperatures during either injection or column elution) could significantly improve the chromatographic response of some drugs. For polar drugs, however, derivatisation was required to sufficiently improve their chromatographic response on the non-polar GC system.

6.2 Practical Considerations

To maximise the number and chemical diversity of drugs which can be detected by the broad drug screening procedure, the extract produced following the extraction of a blood specimen must be appropriate for both underivatised and derivatised GC analyses. The analysis of multiple GC fractions has the advantage that some drugs could be identified in two separate fractions and this would provide strong confirmatory evidence of the presence of a drug in a blood specimen (Section 1.3).

In Section 1.4.2 it was indicated that a comprehensive drug screening procedure which employed benchtop GC/MS operated in SIM mode would require that the analyses should target specific drugs. Consequently, a choice was made as to which drugs would be selected for investigation, given that not all drugs currently available (prescribed and illicit) could be included in the time frame of the present research. Table 1.2 listed over 220 drugs which met at least one of several criteria for inclusion in the drug screening procedure. Not all target drugs listed in Table 1.2 were investigated chromatographically (Chapters 3–5), but their chromatographic behaviour and the likelihood that they would form either butyl or PFP derivatives can be estimated where these untested drugs are structurally similar to target drugs already studied (Section 6.1).

In Chapter 5 it was shown that the majority of PFP derivatives were detected following OC injection as some temperature-sensitive derivatives decomposed at splitless injection. If both OC and splitless injections were to be routinely employed by the broad drug screening procedure, it would require that two different injection ports on the GC be utilised. Consequently, from a practical perspective, all GC fractions could not be analysed in a single automated overnight sequence as column switching either at the detector (dual columns) or injector (single column) would be required. It was necessary, therefore, to compromise on the total number of PFP drug derivatives which could be detected by employing splitless injection routinely for the analysis of all GC fractions.

6.3 Retention and Elimination of Target Drugs

Table 6.1 lists the calculated lowest drug concentration in blood (μg/mL) which could be detected by GC/MS for 177 drugs and their derivatives investigated in Chapters 3–5. This concentration was calculated from the smallest amount of drug/derviative in a pure solution which was detected (Tables 3.2, 3.4, 4.8 and 5.9) and assumes the following conditions:-

1. 100% drug recovery from blood;
2. extraction of drugs from 0.5 mL of blood for those detected underivatised or as PFP derivatives, and from 1.0 mL of blood for those detected as butyl derivatives (Figure 2.1);
3. a 1 μL splitless injection; and
4. a final volume of 50 μL for all underivatised and derivatised blood extracts.

The expected therapeutic range in serum or plasma is listed where a GC peak was detected for either a drug and/or GC artefact, or its butyl or PFP derivatives or reaction artefacts in the majority of instances.[1-6] The exact data between sources often differed but were generally comparable, and were absent for many drug metabolites; a therapeutic range or peak plasma level is listed for 143 drugs and derivatives. The therapeutic ranges listed in Table 6.1 serve only as a guide (Section 10.1) to the drug concentrations that might be expected in blood specimens as:-

1. there is considerable individual variation in response to a drug;
2. pharmacological tolerance can develop with repeated use of the same drug; and
3. different effective ranges are cited[1-6] for different pharmacological actions of the same drug.

Many of the drugs listed in Table 6.1 were chromatographed in both underivatised and derivatised forms. No single GC peak ('x' or 'M') was detected for 8.5% of drugs following chromatography of either their underivatised or derivatised forms (eg. diuretic agents - acetazolamide, chlorthiazide).

Data in Table 6.1 indicate that for 94% of drugs for which a therapeutic range or peak plasma level could be given, the minimum estimated level in blood which could be detected following GC/MS analysis fell within, or below, the reported therapeutic ranges for those drugs. Consequently, should a blood specimen contain these drugs at therapeutic levels, it would be expected that the GC/MS could detect them (assuming 100% recovery from blood). These drugs, therefore, were selected for further study (Chapter 7).

Table 6.2 lists 120 drugs eliminated from further investigation which were 'flagged' in Table 1.2 for inclusion in an automated broad drug screening procedure designed for both clinical and forensic applications. Data in Table 6.1 indicate that, as either no GC peak or multiple peaks were detected for some drugs, the instrument and/or GC conditions were clearly inappropriate for these drugs and their derivatives (if formed) and so they were eliminated from further study (Table 6.2). Similarly, the chromatographic responses of other drugs and drug derivatives listed in Table 6.1 were not great enough to ensure they would be detected in blood specimens at therapeutic levels (the minimum calculated level detected > highest reported therapeutic level). These drugs, therefore, were also eliminated from further study (Table 6.2).

It was important that some drugs for which the minimum estimated level detected was greater than the highest reported therapeutic level were included in a broad drug screening procedure designed for a forensic (toxicological) application (Table 1.2; eg. oxycodone). Although these drugs would not be detected at therapeutic levels their presence at higher (potentially toxic/fatal) blood levels would be possible and, consequently, they were not excluded from further investigation. Haloperidol, however, could not be chromatographed at concentrations equivalent to even fatal blood levels on the non-polar column and GC temperature parameters selected for this study, and was, therefore, excluded (Table 6.2).

Of the approximately 220 drugs selected for inclusion in the broad drug screening procedure (Table 1.2) the selection of some was based on their DDD > 0.3 (Section 1.5 and Table 1.1). The DDD value of these drugs indicated that they were likely to be commonly encountered in the community and included many mineral supplements (calcium, iron), hormonal medications, antibacterial and antifungal agents, and topical or localised (non-systemic) medications. Some of these medications are high molecular weight, relatively involatile compounds and would, therefore, be better suited to analysis by HPLC than GC. It is not unexpected then, that from the data generated in Chapters 3–5, it is estimated that the instrument and/or GC conditions would be inappropriate for these drugs. Similarly, it can be reliably estimated that following either topical or localised application of some medications, their levels in blood would be too low to be detected (Table 6.2).

Similarly, some of the drugs listed in Table 6.2 were excluded from further study on the basis of their reported therapeutic ranges[1-6] and their estimated chromatographic performance. Their estimated chromatographic performance was based on the chromatographic behaviour of structural analogues investigated in Chapters 3–5 and their observed limits of detection. For example, chlorthalidone, indapamide, piroxicam, temoxicam and methylclothiazide were eliminated on the basis that hydrochlorthiazide, chlorthiazide and metolazone could not be chromatographed. These drugs all possess a polar $-SO_2$ group either on an aromatic ring, or as part of a heterocyclic structure. Bumetamide was compared with frusemide and it was estimated that it would not be detected at therapeutic levels given the high detection limit observed for frusemide which is administered in relatively larger doses. Famotidine and metformin were eliminated as cimetidine and ranitidine could not be detected at therapeutic levels.

One hundred and twenty of the drugs listed in Table 1.2 were eliminated from further study. Approximately 40% of these were drugs which were commonly used in the community (as indicated by DDD) but which did not satisfy any of the other selection criteria applied to drugs in Table 1.2. The elimination of these drugs, therefore, is likely to impact most significantly on the clinical application of the automated broad drug screening procedure rather than on its forensic application. In the clinical environment, the presence or absence of these drugs (mineral supplements, hormonal medications, antibacterial and antifungal agents, and non-systemic medications) at therapeutic levels in an individual's blood may be important. Over 106 drugs were retained for investigation of their extraction characteristics from whole blood.

References

1. "Goodman and Gilman's the Pharmacological Basis of Therapeutics."
Hardman J, Limbird L (Eds. in Chief) 9th Edition (1996) The McGraw-Hill Company USA, 1712 - 1792

2. "Disposition of Toxic Drugs and Chemicals in Man."
Baselt R, Cravey R (Eds.) 4th Edition (1995) Chemical Toxicolgical Institute, USA

3. "Therapeutic and toxic drug concentrations."
Uges R; TIAFT (1996) 26,1-34

4. "Melmon and Morrelli's Clinical Pharmacology Basic Principles in Therapeutic"
Melmon K, Morrelli H, Hoffman B (Eds); 1992 3rd ed. 1029-57; McGraw-Hill Inc. USA

5. "A collection of therapeutic, toxic and fatal blood drug concentrations in man"
Stead A, Moffat A; Human Toxicol. (1983) 3, 437-64

6. "Toxicological tables: a compendium of pharmacological, therpaeutic and toxicological data on 136 drugs and chemicals in humans."
Osselton M; TIAFT (1983) 17, 17-33

Chapter 7.

DEVELOPMENT OF A DRUG SCREENING STRATEGY FOR WHOLE BLOOD

7.1 Introduction

7.1.1 General

In Sections 1.3 and 1.4 it was suggested that an automated comprehensive drug screening procedure could realistically expect to satisfy nine requirements and seven of these were related to the extraction of drugs from a whole blood matrix.

In addition, other practical objectives for the drug extraction technique are as follows.

1. The need for drug specificity (excluding endogenous material).
2. The removal of unwanted protein or other endogenous material that would interfere with drug identification.
3. The removal of material that would affect chromatographic performance and reproducibility.
4. Solubilisation of drugs to enable injection under initial chromatographic conditions.
5. Concentration of the drug to within the detection limit of the GC/MS.
6. Stabilisation of the drug to avoid hydrolytic and/or enzymatic degradation.

Consequently, the aim in the development of a drug extraction procedure was to meet as many as possible of the stated requirements and objectives.

7.1.2 The Test Group of Representative Drugs

A drug extraction test mix was employed to evaluate the capabilities of the drug extraction techniques selected for investigation. It was necessary that the test group include drugs.

1. Commonly used in the community (as indicated by DDD).
2. Considered high priority drugs in a forensic application (eg. drugs of abuse; drugs which act directly on the CNS, drugs of toxicological interest).
3. With different physicochemical properties (acidic, neutral and basic).
4. Which represented different pharmacological drug groups (eg, anti-inflammatory or ß-blocking agents).
5. Which represented the different structurally-related drug groups (Section 3.3.1; eg. barbiturates, benzodiazepines).

Table 7.1 lists 33 drugs which were suitable for inclusion in a drug extraction test mix and which satisfied at least one of the five requirements given above. Twenty-five of the drugs were selected (bold text). The first group (1–11) represent the drugs of abuse (DOA). Within this group, amphetamine and methylamphetamine represent typical 1° and 2° amines respectively, and their extraction characteristics may be indicative of the likely recovery from blood of many other drugs with these functional groups. Their extraction characteristics would also be representative of the other phenylethylamine derivatives included in Table 7.1 (ephedrine, MDMA,

pseudoephedrine and phentermine). Similarly, it was anticipated that the extraction of 7-amino flunitrazepam would be similar to that of 7-amino nitrazepam, and that benzoylecgonine, morphine and oxazepam would be representative of other amphoteric drugs.

The second group of drugs (12–26) are the most commonly used drugs in the community (DDD > 5.0; Table 1.1) which could be detected at therapeutic levels by GC/MS. These drugs also represent several pharmacological and structurally-related drug groups as indicated in Table 7.1. In addition, the extraction characteristics of:-

1. naproxen may be representative of other drugs with carboxylic acids functions (including those in Table 7.1; diclofenac and ketoprofen); and
2. paracetamol (and THC-COOH) may be representative of other weakly acidic and neutral drugs.

Each drug in the last group (27–33) is representative of a specific drug group not already represented by another drug listed in Table 7.1. These drugs were the most commonly used drugs within each group (highest DDD; Table 1.1).

Except for felodipine, the chromatographic behaviour of all of the test drugs and/or their butyl or PFP derivatives had been investigated (Chapters 3–5).

7.1.3 Drug Extraction

7.1.3.1 *General*

It was indicated in Chapters 3–5 and Appendix B that drug chromatography was adversely affected as a result of adsorption to 'active sites' in the chromatographic system. Consequently, any routinely employed extraction procedure must be able to not only isolate drugs from blood but minimise the co-extraction of endogenous blood products which may cause chromatographic performance to deteriorate. Contamination of the capillary column and injector of the GC are the most frequent causes of deteriorating performance. In most cases, the contaminating species are involatile residues present in the injected sample.[1] Therefore, the drug extraction technique selected for routine use in the automated broad drug screening procedure will influence the amount of involatile residue present in the injected sample. One study[1] reported that samples extracted by a liquid-liquid extraction (LLE) technique (without additional 'cleanup' steps) contaminated the chromatographic system to a greater extent and much faster than those extracted by a solid-phase extraction (SPE) method. Consequently, the extraction method employed may have a significant effect on the number of GC samples which can be injected before maintenance of the GC system is required.

One of the major interfering substances in blood (and other biological samples) is protein. There are several practical reasons for the removal of proteins from the biological sample prior to analysis:-

1. to inactivate metabolic activity;
2. to remove bound drugs from their protein binding sites;
3. to avoid protein interferences in the identification of drugs (eg, potential formation of artefacts in the hot injector); and

4. to minimise deterioration of the capillary column and reduce contamination and deposits in the injector.

Traditional protein precipitation techniques (eg with trichoroacetic acid) produce supernatants free from excessive contamination but which are extremely acidic. Consequently, acid-labile drugs would be hydrolysed with trichloroacetic acid.[2] In addition, these traditional techniques also cause the co-precipitation of drugs with the protein making them unavailable for extraction.[3] In contrast, a precipitant which produces a 'loose floc' (eg an organic solvent) rather than a dense, compact precipitate will give minimal contamination and minimises drug losses as a result of their co-precipitation with proteins.[3] Water miscible organic solvents such as acetone, methanol, ethanol and acetonitrile, are used to lower the solubility of proteins and precipitate them from solution.[2,3] Organic solvents deproteinise by destroying quaternary and tertiary structures and often release bound drugs.[2] It has been reported that organic solvents in a ratio of 2:1 with the aqueous phase precipitate more than 90% of the protein. The relative order of effectiveness of water miscible organic solvents in precipitating proteins is acetonitrile > acetone > ethanol > methanol which is approximately inversely proportional to their polarity.[4,5]

The major disadvantage of all protein precipitation techniques, however, is the potential loss of drug through adsorption to the precipitate. An alternative to protein precipitation is to separate the protein from smaller analytes through filtration. 'Ultrafiltration'[4] is commonly applied either as pressure or as centrifugal force (1000–2000g). Membranes with a molecular mass cut-off between 10000 and 50000 are chosen. The efficiency of protein removal by ultrafiltration is very high. Compared with protein precipitation, ultrafiltration is a very mild method with little risk of producing artefacts or introducing impurities. A major disadvantage is that certain susceptible drugs may be adsorbed to the filtration membrane itself[4] and only free drug is removed as the drug-protein binding is not ruptured.

Any sample manipulation to isolate drugs from their biological matrix may involve loss of drugs, introduction of impurities, formation of artefacts, a decrease in analytical precision and an increase in analysis time. Since the drug of interest must not be modified or destroyed, the isolation step usually employs a physical distribution of compounds between two immiscible phases. The two most common extraction techniques are LLE and SPE.

7.1.3.2 *Liquid-liquid Extraction*

LLE commonly involves the direct extraction of the biological sample with a water-immiscible solvent. Drugs can be removed from their biological matrix (including interfering endogenous products) by adjusting pH (if necessary) and extracting the un-ionised form with the organic solvent. The isolation of the drugs is achieved by partitioning between the organic and aqueous phases and an equilibrium distribution is established between the two phases. The aim of the technique is to create conditions which preferentially distribute the drug into the organic phase and the distribution will be influenced by the following:-

1. the pH of the aqueous phase;
2. the pKa of drugs; in general, two pH units above the pKa for basic drugs and two pH units below the pKa for acidic compounds results in optimal extraction;[3,6]

3. the extracting solvent (the partition co-efficient between aqueous phase and the particular solvent); and
4. the ratio of the volumes of organic to aqueous phases; if drug recovery is low, either successive extractions, or extraction with a large excess of solvent, improves recovery.[2,4,7]

In general, lipophilic drugs pass from water into non-polar solvents while polar drugs tend to favour water due to their potential for H-bonding with water molecules. Increasing the polarity of the extraction solvent, therefore, increases the solubility of the polar drug in the extraction solvent.[8]

Early techniques involved the use of non-selective solvents (eg diethyl ether, chloroform) which extracted lipophilic material such as lipids and fatty acids as well as the drug of interest. These materials both contaminated the chromatographic system and interfered with the peaks of interest during their chromatography.[9] One of the earliest reported selective extraction solvents was *n*-butyl chloride which also gave high recoveries for basic drugs.[9]

The solubility of many organic substances in water is decreased by the presence of dissolved inorganic salts.[3,10] For example, buffer solutions both adjust the pH of the aqueous phase and decrease the solubility of drugs in the aqueous phase by this 'salting out' effect. The 'salt-solvent' pairing has the added benefit of decreasing emulsion formation[11] but a disadvantage is that interfering species are also co-extracted.[9]

7.1.3.3 *Solid-phase Extraction*

SPE works on the principle of selective retention of drugs on cartridges containing sorbents (100–1000 mg) similar to those used in HPLC. In this process, the sample passes over the stationary phase and the drugs are adsorbed if they have a greater affinity for the stationary phase than the sample matrix, but, unlike chromatographic separation, the drugs are completely retained on the stationary phase rather than retarded. Retained drugs can be removed at a later stage by eluting with a solvent (if the drugs have greater affinity for the solvent than the stationary phase). Several reviews have explored the analytical variables associated with solid-phase extraction.[7,9,12] SPE provides very high theoretical recoveries due to the large capacity of the sorbent bed. A typical SPE consists of four steps as follows:-[7,9,12]

1. The sorbent bed is conditioned (solvent + aqueous phase) to prepare the dry/stored cartridge to receive the sample, to improve reproducibility of drug retention and to rinse away materials contaminating the sorbent.
2. The sample is applied to the sorbent and drugs are retained together with undesirable matrix constituents; unretained matrix constituents pass through the sorbent and are discarded.
3. The cartridge is rinsed with a weak solvent to remove some of the undesirable matrix constituents.
4. A sufficiently strong solvent selectively elutes the drugs leaving undesirable components retained on the sorbent.

Therefore, drugs are selectively isolated from the sample matrix with minimal contamination from interfering species.

During cartridge conditioning, the siloxane structure of the silica binds a permanent layer of water molecules. Protruding from the water layer are the bonded silica chains and interdispersed between them are molecules of water and the conditioning solvent. The higher the organic content of any washing, conditioning or eluting solution which is applied to the cartridge, the more extended are the chains.[7] Consequently, it might be expected that the bonded silica chains are more extended during sample elution than during sample application. The analyte can be bound to the sorbent by a number of different mechanisms which have been reviewed.[7] The different mechanisms of retention or elution result from intermolecular forces between the analyte, the active sites on the surface of sorbent, and the liquid phase (sample matrix and subsequent washing and eluting solutions).[12]

The sorbents employed in SPE are generally similar to those used in column liquid chromatography: nonpolar, polar and ion exchange phases each designed for particular analytes. 'Mixed phases' are also available which possess various functional groups of different character attached to the same adsorbent particle which can isolate drugs (polar and non-polar) over the entire pH range.[12] For example, Bond Elut Certify (Varian) and CleanScreen (Worldwide Monitoring) are mixed-mode bonded silica gel SPE cartridges which contain both nonpolar (hydrophobic) and cation exchange functional groups specifically designed for broad drug screening.[13,14]

Much of the literature addresses SPE of drugs from urine and serum. As a result of the tightly packed sorbent within the cartridges, the analysis of drugs in blood and tissues by SPE poses an analytical problem not encountered with LLE due to the potential for clogging of the cartridge. In addition, many drugs are present inside red blood cells and/or are bound to them and must be released prior to extraction.[14] The preparation of serum or plasma from haemolysed whole blood is not possible and, therefore, whole blood requires pretreatment prior to SPE. Pretreatment involves either protein precipitation, or dilution of the blood with a suitable buffer solution.[15] It has been previously indicated that protein precipitation leads to reduced drug recovery due to co-precipitation of drugs. A comparison of blood pretreatments prior to SPE has been reported.[14] It was found that drug recovery from whole blood with SPE was greater following sonication of the blood sample and dilution than after protein precipitation.

7.1.3.4 *Selection of Drug Extraction Techniques*

It was indicated in Section 1.4.1 that there had been a vast amount of literature published in the area of drug analysis in biological materials. Many procedures were designed to extract a specific drug or drug group rather than to be broadly applicable across drug groups. Similarly, the volume of literature devoted to the subject of drug analysis via chromatographic techniques indicated that it was constantly being explored. Consequently, published extraction techniques were considered for investigation if they possessed at least two the following characteristics.

1. A range of chemically diverse drugs were extracted.
2. Drugs were extracted from blood (including serum and plasma) or the technique could be easily modified to extract drugs from blood.
3. Drugs were extracted from a single, limited volume sample (< 3 mL).

4. The technique was routinely employed in either a forensic or clinical laboratory.
5. The extraction efficiency for a wide range of drugs was indicated.
6. Drug detection was by a chromatographic method of analysis (GC in particular).
7. The technique was either very selective, or a 'cleanup' step was incorporated, which ensured that interfering and/or contaminating endogenous material was removed prior to analysis.
8. Recovery data were given for some drugs included in the extraction test mix (Table 7.1).

Table 7.2 gives limited details of 23 published drug extraction techniques selected for consideration. Both LLE and SPE techniques are included. SPE techniques which employed the mixed-mode phases designed for broad drug screening (extraction of acidic (A) neutral (N) and basic (B) drugs) were selected in preference to those which employed the more traditional bonded phases.

Data in Table 7.2 indicate the following.
1. Details of the identity and number of drugs recovered by the technique was often incomplete.
2. Recovery data was generally not supplied for all drugs investigated.
3. LLE techniques were more common pre-1990 and SPE techniques (with mixed-mode sorbents) more common post-1990.
4. LLE techniques were routinely used in forensic and clinical laboratories.
5. LLE techniques employed either a selective extraction solvent (*n*-butyl chloride) or a non-selective extraction solvent coupled with an extract 'cleanup' step (eg. back extraction) prior to analysis.
6. LLE techniques often did not investigate, or did not recover, drugs of abuse (eg. amphoteric drugs; benzoylecgonine, morphine, oxazepam).
7. LLE techniques developed for blood were generally not able to extract A, N and B drugs from the same aliquot.
8. 'Salting out' techniques (with strong/saturated solutions of inorganic salts) were commonly employed in LLE techniques.
9. SPE techniques which analysed biological fluids other than whole blood were most common.
10. SPE techniques were able to extract A, N and B drugs from a single aliquot of sample.
11. SPE techniques were drug specific and did not generally employ any 'cleanup' steps prior to chromatographic analysis.
12. The amphoteric drugs were sometimes difficult to elute from the cartridge during SPE and required more polar eluants.

The number of published extraction methods for consideration was further reduced by selecting methods from Table 7.2 which:-
1. were in routine use in either forensic or clinical laboratories;
2. extracted a large number of the drugs listed in Table 7.1 with minimal manipulation (eg no protein precipitation, back extraction and pH adjustments);
3. were developed in whole blood and analysed by GC; and
4. represented both LLE and SPE techniques.

Consequently, methods 8, 17 and 21 were selected for further investigation. In all future references to these methods in the chapter, numbers '8', '17', and '21' will identify them.
Method 8 was designed for the extraction of acidic and neutral drugs (Table 7.2) and was routinely employed in a toxicology laboratory but neither the number of samples which had been analysed by this method, nor how long it had been in routine use by the laboratory, was stated.[23] As the method employed a 'salting-out' step (Table 7.2) it might be expected that emulsion formation and lipid contamination were minimised, and drug recovery was improved from that with ethyl acetate alone. As there was no significant pH adjustment, protein was not excessively denatured/precipitated and the blood remained fluid.[23] Consequently, it might be anticipated that drug loss through sample pretreatment was minimal and all the sample remained available to the extraction solvent. There was no 'cleanup step' incorporated into the procedure despite the use of a non-selective extraction solvent;[23] therefore, some modification of the method would be likely if it were adopted for routine use in the present study.

Method 17 employed mix-mode stationary phase cartridges to extract A, N and B drugs from plasma (and whole blood; Table 7.2 Method 23). Minimal sample pretreatment was required (sonication and dilution) prior to SPE.[32] Successive pH adjustments of the cartridge allowed the elution of A/N drugs first followed by B/N drugs.[32] The cartridge washing prior to drug elution removed some blood proteins and other interfering matrix constituents.[32] The use of relatively weak solutions to alter pH ensured that any blood protein retained on the cartridge was not precipitated, otherwise clogging of the cartridge would have resulted. It was indicated that amphoteric drugs were not eluted from the sorbent but more polar eluants did elute these drugs (Table 7.2; methods 19, 20 and 22). Consequently, some modification of the eluants used in this method would be expected it were to successfully extract the majority of drugs in the extraction test mix (Table 7.1).

Method 21 was similar to Method 11 in design and in the range of drugs extracted, but was more extensively tested in forensic laboratories (Table 7.2). No sample pretreatment was employed.[36] Reference was made to the chromatographic performance of the GC system following the repeated injection of forensic samples with 100 injections being made before any maintenance was required (replaced injector liner).[36]

7.2 Methods

7.2.1 General

Refer to "General Procedures" (Chapter 2). In particular, for HC marker solution refer to Section 2.2.5.5 and 2.2.5.6; for reference drug solutions refer to Section 2.2.5.14; for instrumentation refer to Section 2.4.1; for SIM acquisition parameters refer to Section 2.4.3.1; for gas chromatographic parameters with the wider bore column refer to Section 2.4.2; for the butylation of drugs refer to Sections 2.2.3 and 2.2.5.4 for details of reagents, to Section 2.3.2 for apparatus employed and to Figure 2.4 for the derivatisation procedure; for the pentafluoropropionylation of drugs refer

to Section 2.2.4 for details of reagents, to Section 2.3.2 for apparatus employed and to Figure 2.5 for the derivatisation procedure.

Blank blood was obtained from the Government Analytical and Forensic Laboratory (GAFL). It was 2 year old blood obtained for alcohol determination under the Road Safety (Alcohol and Drugs) Act which had been stored at 4°C in bottles with sodium fluoride and potassium oxalate as preservative and anti-coagulant, respectively. Thirty to 40 individual blood samples were bulked together and used in all experiments. Although the individual blood specimens may not have been drug-free, by bulking a large number of specimens together, the final drug concentration would become negligible.

7.2.2 Drug Extraction Test Mix

Refer to Table 7.1 for drugs included in the test mix. A mixed drug solution (10 μg/mL) in methanol was prepared.

7.2.3 Mixed Deuterated Internal Standard Solution

Deuterated drug standards (amitriptyline-d3, amphetamine-d6, benzoylecgonine-d3, codeine-d3, methadone-d3, methylamphetamine-d6, morphine-d3, nordiazepam-d5, oxazepam-d3, temazepam-d5, and THC-COOH-d3) were obtained from Alltech Australia. A mixed drug solution (10 μg/mL) was prepared in methanol.

7.2.4 Liquid-liquid Extraction

7.2.4.1 *General*

Refer to Sections 2.2 and 2.3 for details of reagents and apparatus. Refer to Figure 2.2 for the extraction procedure. Method 8[23] and 21[36] (Table 7.2) were investigated but with some immediate modifications as follows.

1. The volume of blood extracted was 1.5 mL and pH adjustments were made with correspondingly increased volumes of buffer (Method 8; 0.75 mL saturated ammonium chloride and Method 21; 1.5 mL 2M TRIS).
2. Extraction was performed with two aliquots of the solvent rather than one to improve drug recovery (Method 8; 2 x 5 mL ethyl acetate and Method 21; 2 x 3 mL *n*-butyl chloride).

During investigation of LLE, the following parameters in Methods 8 and 21 were varied.

7.2.4.2 *Variations to Method 8.*

1. Volume of ethyl acetate (3 and 5 mL);
2. *n*-Butyl chloride was substituted for ethyl acetate as the extraction solvent; (this variation is indicated by 'BC' in future tables and discussions);
3. The aqueous phase was made more acidic (pH 5) with 150 μL acetate buffer; (this variation is indicated by 'A8' in future discussions);
4. Methods 8 and 21 were combined to investigate the feasibility of A, N and B drug extraction from a single aliquot of blood (Section 7.2.4.3);
5. One of two 'cleanup' steps ('AH' or 'E') was incorporated following drug extraction (1 mL of blood + 0.5 mL of saturated ammonium chloride were

extracted with 2 x 5 mL aliquots of ethyl acetate); only underivatised and butylated blood extracts were analysed.

'AH':- Involved the partitioning (x 3) of drugs and contaminants between hexane saturated with acetonitrile (0.5 mL) and acetonitrile (50 μL)[24] but, in the present study, partitioning was performed only twice (Section 2.5.1.4). The top hexane layer was aspirated to waste.
'E':- Involved the partitioning of drugs and contaminants between water miscible and immiscible solvents.[21] The extract residue was redissolved in 7 mL of hexane:ethanol (10:4). Water (0.5 mL) was added and the phases separated. The top hexane layer was aspirated to waste.

7.2.4.3 *Variations to Method 21.*

1. The aqueous phase was made more basic (pH 10.6) with 50 μL 20% NaOH; (this variation is indicated by 'B21' in future tables and discussions);
2. Methods 8 and 21 and Methods 8 and 'B21' were combined to investigate the feasibility of A, N and B drug extraction from a single aliquot of blood; (these variations are indicated by '8 + 21' and '8 + B21' in future tables and discussions); the extraction procedures were combined as follows:-

   The blood was extracted with 5 mL of ethyl acetate (Method 8); the pH of the blood was then adjusted with 2 mL 2M TRIS (pH 9.4; Method 21) or 2 mL 2M TRIS + 50 μL 20% NaOH (pH 10.6; Method 'B21') and extracted with 5 mL of *n*-butyl chloride. The organic phases were combined and evaporated under nitrogen prior to the addition of HC markers and the internal standard solution (Section 7.2.6).

7.2.5 Solid-phase Extraction

Refer to Sections 2.2 and 2.3 for details of reagents and apparatus. Refer to Figure 2.3 for the extraction procedure. Method 17[32] (Table 7.2) was investigated but with some immediate modifications as follows.

1. All solutions/eluants were passed through the cartridge without the application of a vacuum which was only applied during the cartridge drying stage.
2. Blood (1.5 rather than 1 mL) was applied to the extraction cartridges without a corresponding increase in the volume of phosphate buffer used to dilute it.
3. Eluants were applied in 2 x 2 mL aliquots rather than a single 4 mL aliquot.
4. The basic/neutral drug eluant was 5% ammonia in dichloromethane/isopropyl alcohol (80:20) rather than 2% ammonia in ethyl acetate.

During development of a drug extraction strategy for the automated broad drug screening procedure, the following parameters in modified Method 17 were varied.

1. Elution of basic/neutral drugs with a single 2 mL aliquot of 5% ammonia in methanol (this variation is indicated by 'MEOH' in future tables and discussions).
2. Separation of the pH adjustment of the cartridge from the elution of basic/neutral drugs; the pH of cartridge was adjusted with 200 μL of a dilute ammonium hydroxide solution in methanol (25% ammonium hydroxide:methanol - 1:20) and was followed by the elution of basic/neutral drugs with dichloromethane/isopropyl

alcohol (80:20); (this variation is indicated by 'NH_3' in future tables and discussions).

7.2.6 Evaluation of Drug Extraction Procedures.
Three extracts for GC were prepared following each extraction procedure - underivatised (U), butylated (B) and pentafluoropropionylated (P). Each extract was equivalent to 0.5 mL of extracted blood. Each extract contained the drug extraction test mix, the HC marker solution and the mixed deuterated internal standard solution.

The drug extraction test mix (150 µL) was added to glass culture tubes and reduced under nitrogen to approximately 20 µL. Blank blood (1.5 mL) was added to the culture tube and allowed to equilibrate for approximately 15 min. Tubes of blank blood containing no drug test mix were also prepared. The blood (spiked and blank) was extracted by the test extraction procedures (Sections 7.2.4 and 7.2.5). The HC marker solution (150 µL) was added to the resulting extraction solvents (LLE) or eluants (SPE) which were then divided by eye into three approximately equal aliquots. Mixed deuterated internal standard solution (50 µL) was added to each of the three aliquots. Each aliquot was reduced to dryness under nitrogen prior to either the addition of the injection solvent (U) or derivatisation (B, P). Unextracted reference solutions were prepared concurrently (U_{std}, B_{std} and P_{std}) for comparison with extracts generated from the test extraction procedures and each contained 50 µL of the drug extraction test mix, the HC marker solution and the mixed deuterated internal standard solution. The final volume of all extracts and reference solutions for GC was 50 µL.

The peak area ratio (Section 2.6.1) was obtained for all test drugs in both extracts and reference solutions and enabled a direct comparison (as a percentage) of extracted drugs with drugs in reference solutions. For those drugs with corresponding deuterated internal standards, the peak area ratio was determined from the absolute peak areas of the drug and deuterated drug rather than from the closest eluting HC marker.

Experiments testing each extraction technique were performed in triplicate on different days and the mean percentage of drug recovered by the technique was calculated together with the standard deviation from the mean (SD).

7.3 Results

7.3.1 Liquid-liquid Drug Extraction
The recovery from blood of a drug extraction test mix (Section 7.1.2) was investigated employing variations to two LLE techniques (Methods 8, and 21; Sections 7.2.4 and 7.2.6).

Table 7.3 shows the percentage of drug recovered (mean, SD) by each of the LLE techniques investigated. The drugs of the extraction test mix were analysed in either their underivatised or derivatised forms. In general, the underivatised drugs can be considered as weakly acidic, neutral and basic drugs; the butyl derivatives are formed from acidic drugs (Chapter 4); and the PFP derivatives are formed from basic drugs

(Chapter 5). Within these groups, however, there is an overlap of drug chemistries (eg. amphoteric drugs). Different extraction methods are indicated by the abbreviated labels assigned to them in Section 7.2.4 and these are summarised in Figure 7.1.

Data in Table 7.3 indicate the following.

1. The greatest number of drugs was recovered by Methods 8, '8 + 21' and 8 + B21' but the recovery of basic drugs was higher by the combined techniques (eg. amphetamine, methylamphetamine, metoprolol).
2. The least number of drugs was recovered by Methods 21 or 'B21'.
3. Benzoylecgonine and captopril were not recovered by any method tested.
4. Morphine was recovered only by Methods 8, '8 + 21' and 8 + B21' but recoveries were poor.
5. Acidic drugs were not recovered by Methods 21 or 'B21' unless they were performed in combination with Method 8.
6. Strongly basic drugs (amphetamine and methylamphetamine) were best recovered by Methods 21 and 'B21'; Method 'B21' recovered basic drugs relatively better than Method 21, however, neither method recovered acidic drugs.
7. Choice of solvent was important for the extraction of drugs by Method 8; for example, recovery of drugs by Methods 8 and 'BC' were comparable for the majority of basic drugs (excluding metoprolol) but, for acidic drugs (paracetamol, theophylline, naproxen and frusemide) recovery was better by Method 8 than 'BC'.
8. Recovery of drugs by Methods '8 + 21' and 8 + B21' were comparable.
9. Apart from the extraction of morphine and the strongly basic drugs, neither combined extraction technique recovered drugs significantly better than Methods 8 and 21 alone.

During investigation of the LLE techniques, the following practical observations were made.

1. During initial investigations of Method 8, blood was extracted with 2 x 3 mL aliquots of ethyl acetate. However, emulsions were quickly formed with the blood precipitating into thick lumps which adhered to the walls of the culture tubes. Following extraction, the tubes were very dirty and difficult to clean. There was difficulty separating the aqueous and organic phases by centrifugation and often these tubes would have to be shaken and spun down again to achieve separation. Increasing the volume of ethyl acetate from 3 to 5 mL alleviated all of these problems. No emulsions were formed and the blood remained relatively fluid at end of the extraction process. There was no difficulty in separating the phases by centrifugation and the glassware was significantly cleaner. All subsequent experiments employed 5 mL aliquots of ethyl acetate. Following extraction, ethyl acetate was straw coloured and, when concentrated, was strongly yellow (presumably due to co-extraction of endogenous lipids, etc).
2. During the investigation of Method 'A8' (acidification of the blood/ammonium chloride solution prior to extraction) the pH of the solution was decreased from 7 (Method 8) to 5. When the acidified solution was extracted with ethyl acetate, emulsions were immediately formed which coated the walls of the culture tubes

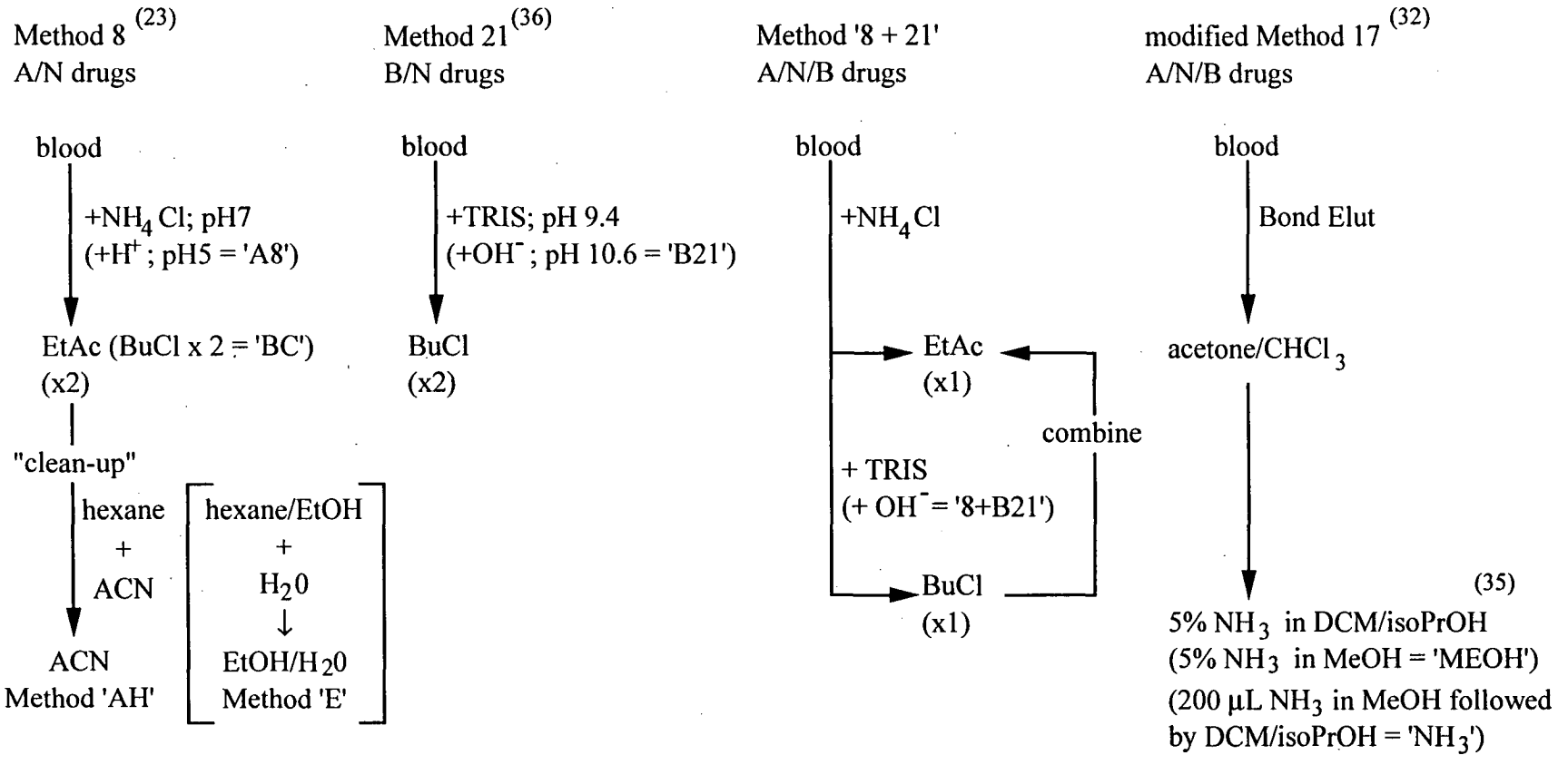

Figure 7.1: A schematic representation of extraction procedures and their variations described in Sections 7.2.4 and 7.2.5.

EtAc	=	ethylacetate	MeOH	=	methanol
BuCl	=	*n* - butylchloride	$CHCl_3$	=	chloroform
EtOH	=	ethanol	DCM	=	dichloromethane
ACN	=	acetonitrite	isoPrOH	=	isopropylalcohol

and had a very viscous almost 'oily' appearance. On centrifugation, the blood formed a very solid precipitate and the solvent was discoloured red-brown. Exchanging the acetate buffer for a citrate buffer (0.1M; pH 5) did not improve the appearance of the blood or solvent. As a result of these problems Method 'A8' was abandoned.

3. Methods which employed *n*-butyl chloride (Methods 21, 'B21' and 'BC) were always free of emulsions, no blood adhered to the glass walls of the culture tubes and the aqueous and organic phases separated rapidly on standing. *n*-Butyl chloride was more quickly evaporated under nitrogen than was ethyl acetate and the concentrated solvent was not significantly discoloured.
4. When *n*-butyl chloride was substituted for ethyl acetate (Method 'BC'), it was observed that the organic and aqueous phases did not really ever appear to mix but moved over each other during extraction as though they repelled each other. Much more vigorous shaking was required rather than the 'end-over-end' mixing employed for all other extraction techniques.
5. Cholesterol was the major contaminating peak in chromatograms from blank blood extracted produced by Methods 21 and 'B21'. A cholesterol peak was present but reduced in chromatograms from blood extracts produced by Methods 8 and 'BC'.
6. The cholesterol peak significantly interfered with the detection and integration of butyl-THC-COOH and thioridazine peaks.
7. During the investigation of Method 'B21' (basification of the blood/TRIS solution prior to extraction) the pH of the solution was increased from 9.5 (Method 21) to 10.6. When the basified solution was extracted with the 3 mL aliquot of *n*-butyl chloride emulsions were formed immediately. This problem was alleviated by increasing the volume of *n*-butyl chloride to 5 mL. The cholesterol contaminant was much reduced in chromatograms from Method 'B21' compared with Method 21 but continued to interfere with integration of thioridazine and butyl-THC-COOH peaks.
8. Chromatograms from blood extracts produced by the combined extraction techniques ('8 + 21' and '8 + B21') contained more non-drug related peaks (presumably endogenous blood products) which interfered with the detection and/or integration of drugs from the extraction test mix compared with either Methods 8 or 21 alone. These were apparent in the early part of chromatograms for each of the extracts, but in particular those of butylated extracts.
9. During investigation of the LLE methods, it was quickly apparent that the concurrent chromatography of extracted blood products with some of the underivatised drugs of the test mix improved their chromatographic response from those observed for U_{std} (Section 7.2.6); particularly the responses of promethazine, temazepam and the high boilers (thioridazine and verapamil). However, with repeated injection of blood extracts, and in particular of PFP extracts, the peak areas of many underivatised drugs were significantly reduced; verapamil, thioridazine, temazepam (practically disappeared) nordiazepam, codeine, phenobarbitone.
10. During investigation of the combined extraction techniques, it was always observed that emulsions formed during extraction with the second aliquot of solvent (*n*-butyl chloride) and that the absolute peak areas of temazepam and

deuterated temazepam were significantly reduced compared with any other method.

11. The absolute peak areas of THC-COOH and deuterated THC-COOH in blood extracts from all methods were significantly reduced from those of B_{std} (Section 7.2.6).
12. The glass wool of the injector was discoloured at the end of the GC/MS sequence regardless of the extraction technique employed. Chromatographic performance of susceptible underivatised drugs could not be returned to that observed at the beginning of the sequence without replacing the liner with a clean liner and glass wool. The chromatographic performance of some drugs was improved following priming of the clean liner (by repeated injection of the performance test mix) and conditioning with 1 to 2 injections of an underivatised blood extract.
13. When the SD recorded for a drug was large (Table 7.3), this was generally as a result of poor integration caused either by a closely eluting interfering peak (eg. early eluting butyl derivatives), or the drug peak tailing out of the time window over which it was monitored (eg. nordiazepam and thioridazine).

It was concluded that Method 8 was the most effective extraction technique for the recovery of the majority of drugs in the test mix with the least practical problems, particularly if extract cleanliness could be improved. Consequently, drug recovery by Method 8 was investigated following the incorporation of two different 'cleanup' steps ('AH'[24] and 'E'[21]; Section 7.2.4.2 and Figure 7.1).

Table 7.4 shows the percentage of drug (mean, SD) recovered following extraction of 1 mL of blood by Method 8 with and without 'cleanup' steps incorporated. It also shows the mean percentage of drug recovered by Method 8 when 1.5 mL of blood was extracted (same data as shown in Table 7.3 and included to permit easy comparison). Naproxen is absent from the drugs listed as it was found that the 'blank' blood used in these experiments contained a significant amount of naproxen. Data in Table 7.4 indicate the following.

1. No large differences in drug recovery were observed whether 1.0 or 1.5 mL of blood was extracted by Method 8. The recovery of codeine, tolbutamide, theophylline and frusemide, however, appeared to be slightly improved when only 1.0 mL of blood was extracted.
2. No large differences in the recovery of the test drugs (except THC-COOH) was observed following extraction by Methods 'AH' or 'E' from that observed by Method 8.
3. Drug recovery across the extraction test mix was most variable by Method 'E' (highest value recorded for the sum of SD).
4. The recovery of benzodiazepines (nordiazepam, temazepam and oxazepam) and weak acid drugs (paracetamol, theophylline) was marginally lower following extraction by Methods 'AH' and 'E' from that observed by Method 8.
5. The recovery of THC-COOH was significantly reduced following extraction by Method 'AH' from that observed by Method 8.

During investigation of drug recovery by the Methods 8, 'AH' and 'E', the following practical observations were made.

1. The addition of 0.5 mL of water to the hexane/ethanol organic phase (Method 'E') saw the very rapid re-partitioning of the three solvents and cloudiness developed in the newly formed ethanol/water phase. The evaporation of the water/ethanol mixture under nitrogen was very time consuming compared with any other evaporation step tested (both LLE and SPE methods). Acetone was added (approximately 2 mL) to increase the rate of evaporation (azeotrope formation). In addition, the volume of water added was reduced:- the minimum volume of water required to cause the repartitioning of the three solvents was found to be approximately 0.15 mL (rather than 0.5 mL).
2. Following the addition of the GC injection solvent (Section 2.2.5.7), the underivatised extract from Method 'E' was cloudy with either residual water or extracted water soluble products.
3. During investigation of Method 'AH', it was observed that the 50 µL of acetonitrile was very yellow in colour. Following mixing (x 2) with hexane, the hexane took on an obvious yellow discolouration but the acetonitrile still retained some colour. The drugs extracted from the blood were dissolved in the acetonitrile. There was a danger, therefore, that some portion of the acetonitrile would be aspirated and lost if all the hexane was removed. Therefore, some residual hexane always remained.
4. The cholesterol peak observed in chromatograms from blank blood extracted by Method 8 was either significantly reduced or absent in chromatograms from Methods 'E' and 'AH' respectively. In addition, early eluting peaks observed in chromatograms from Method 8 were absent in those of Method 'AH' and reduced in those of Method 'E'.
5. The absolute peak areas of temazepam and deuterated temazepam in chromatograms from Method 8 were significantly reduced compared with those in Methods 'AH' and 'E'; their peak areas were greatest in chromatograms from Method 'AH'.
6. Visual inspection of the underivatised and butylated blood extracts prior to GC analysis showed that those produced from Method 8 were the most discoloured (yellow), and those from Method 'AH', the least.

Following the evaluation of the different LLE techniques, it was concluded that Method 'AH' offered the best LLE drug screen. It satisfied four important criteria as follow.

1. The majority of drugs from the extraction test mix were recovered.
2. There were fewer practical problems encountered with the extraction of blood and the preparation, derivatisation and GC analysis of extracts.
3. Clean extracts were produced which may extend the period of time over which acceptable chromatographic performance of the GC system can be maintained.
4. There was a minimum of interfering peaks (eg. cholesterol and early eluting peaks) in chromatograms from underivatised and derivatised extracts.

7.3.2 Solid-Phase Drug Extraction

Data in Table 7.5 show the percentage of drug recovered (mean, SD, n=3) by each of the SPE techniques investigated. The drugs of the extraction test mix were analysed in either their underivatised or derivatised forms. Different extraction methods are

indicated by the abbreviated labels assigned to them in Section 7.2.5 and summarised in Figure 7.1.

Data in Table 7.5 indicate the following.

1. Captopril was not recovered by any extraction technique tested.
2. Recovery was poor for strong acids (naproxen, frusemide) and some weakly acidic drugs (paracetamol, theophylline and tolbutamide).
3. Large SD values (> 10%) were associated with the recovery of some drugs by one technique but not another (eg. thioridazine, verapamil, phenytoin).
4. The variability in recovery across the extraction test mix by each method was comparable (similar values recorded for the sum of SD).
5. There were no large differences observed in the recovery of any drug by the various methods tested except for benzoylecgonine and metoprolol; there was less benzoylecgonine recovered by Method 'NH_3' than by the other two methods in which ammonia was incorporated into the B/N drug eluant; there was less metoprolol recovered by Method 'MEOH' than by the other two methods in which dichloromethane/isopropyl alcohol was the B/N drug eluant.
6. Recovery was consistently high for all B/N drugs.
7. All amphoteric drugs were recovered by all methods tested (morphine, oxazepam and benzoylecgonine).

During investigation of SPE techniques, the following practical observations were made.

1. Following the application of the blood to the cartridge, the packing was saturated with the blood/buffer solution and discoloured red. The 1 mL water wash of the cartridge effectively removed residual blood/buffer solution from the packing and the packing was discoloured pink/brown. The application of the A/N drug eluant removed still further material from the packing which was discoloured tan following application of 2 x 2 mL aliquots of acetone/chloroform (1:1).
2. The application of the ammoniacal methanol B/N drug eluant effectively stripped the packing of all colour (Method 'MEOH').
3. With the application of the pretreated blood sample, cartridges always appeared by eye to be evenly wetted. With subsequent applications of either sorbent washes or eluants, however, some cartridges appeared to be unevenly wetted by these solutions. In some cases, it appeared that portions of the cartridge had never been exposed to the washes and eluants.
4. The combined eluants from Methods 'MEOH' and 'NH_3' were discoloured yellow/brown compared with those from Method 17.
5. The time required to evaporate the combined eluants was greatest for Method 'MEOH' (despite a smaller volume of B/N drug eluant) and least for Method 17.
6. It was difficult to completely dry the combined eluants from Methods 'MEOH' and 'NH_3' under nitrogen prior to their derivatisation as each contained a noticeable amount of residue which was absent from Method 17. Consequently, evaporation times were longer for these techniques than for Method 17.
7. With the addition of PFPA to the residue from Method 'MEOH', fumes developed and the final extract was dark brown compared with those of PFP extracts from either Methods 17 or 'NH_3'.

8. Following sequential GC analysis of a series of extracts produced by Methods 17, 'MEOH' and 'NH_3', the glass wool of the injection port liner was very dirty (compared with LLE experiments) but the 'performance test mix' (Sections 2.2.5.10 and 2.6.2) still indicated acceptable performance of the GC system; phenobarbitone, temazepam and promethazine were still apparent, although the absolute peak areas of temazepam, thioridazine and verapamil were reduced.
9. The absolute peak areas of temazepam and deuterated temazepam in blood extracts from all methods were comparable with those in U_{std} (Section 7.2.6).
10. The absolute peak areas of THC-COOH and deuterated THC-COOH in blood extracts from all methods were reduced compared with those of B_{std} (Section 7.2.6).
11. A cholesterol GC peak was either reduced or absent in chromatograms from blood extracts produced by each method and did not interfere with the integration of any drug peak.

Following the evaluation of the different SPE techniques, it was concluded that Method 17 (modified; Section 7.2.5) offered the best drug screen. Although there were no large differences in the recovery of the majority of drugs by the three tested methods (except benzoylecgonine and metoprolol), and all extracts were relatively clean (reflected by the subsequent chromatography of the 'performance test mix'), there were differences in the practical problems associated with each technique. Method 17 satisfied the same four criteria met by Method 'AH' in Section 7.3.1

7.3.3 Drug Recovery by the Selected Extraction Techniques

The suitability of the LLE (Method 'AH') and SPE (modified Method '17') methods to broad drug screening was further evaluated by measuring their capacity to extract all of the 106 target drugs retained for further study in Section 6.3. Working drug solutions were prepared from reference drug solutions (Section 2.2.5.13) and spiked into whole blank blood. Where drugs were able to be detected in more than one extract (as underivatised drugs or as butyl or PFP derivatives), recovery data were obtained for each (eg. clobazam, desipramine, diclofenac). Each extract was equivalent to 0.5 mL of whole blood. The percentage of drug recovered (mean, SD, n=3) was determined on different days by both methods (Table 7.6). The chromatographic response of some drugs and drug derivatives was affected by co-chromatographing extracted material. Consequently, recovery data for these drugs was determined by redissolving U_{std}, B_{std} and/or P_{std} (Section 7.2.6) in a blank blood extract so that both spiked blood sample and reference were chromatographed with similar extracted material.

Data in Table 7.6 indicate the following.

1. Forty-one percent of the tested drugs were similarly recovered by each technique (Table 7.7).
2. Thirty-eight percent of the tested drugs were better recovered by SPE than LLE (Table 7.8).
3. Twenty-one percent of the tested drugs were better recovered by LLE than SPE (Table 7.9).
4. Unusual recovery data was determined for 3 drugs (nifedipine, quinine and quinidine).

5. The recovery of 88% of tested drugs by either SPE or LLE (or both) was greater than 75%.
6. The recovery of 51% of tested drugs by either SPE or LLE (or both) was greater than 90%.
7. The recovery of 6% of tested drugs by either SPE or LLE (or both) was less than 30%, and all were acidic drugs.
8. Baclofen and captopril were not recovered by SPE or LLE techniques.
9. Some drugs were recovered only by SPE (LLE recovery <10%); atenolol, disopyramide, ephedrine, phentermine, prednisolone, sotalol.
10. Some drugs were recovered only by LLE (SPE recovery <10%); chlorpropamide, diflunisal, naproxen and nicotinic, salicylic and valproic acids.
11. Strong acids were not recovered by SPE and poorly recovered by LLE; nicotinic acid (<10%), salicylic acid (18%) and valproic acid (21%).
12. The sum of SD (Table 7.6 - Note 4) associated with the recovery of all test drugs was greater following SPE than LLE.

Data in Table 7.6 clearly indicate that if a broad drug screen were based only on the LLE method several important drugs of abuse (amphetamine-like drugs, benzoylecgonine, morphine, THC and THC-COOH) would be excluded. Similarly, a broad drug screen based only on the SPE method would exclude several drugs which are either prevalent in the community or of toxicological interest (eg. frusemide, naproxen and other anti-inflammatory drugs, paracetamol, phenytoin, theophylline, salicylic and valproic acids). Consequently, the most comprehensive drug screening strategy would be attained by applying both extraction techniques to the same sample of blood and combining their results.

The design of the drug screening strategy illustrated in Figure 2.1 was based on data in Tables 7.6–7.9 (Section 7.4.2). Four GC/MS (SIM) methods (Tables 2.1–2.4) were created for the acquisition of *m/z* ion pairs which would identify target drugs in each extract ('L', 'S', 'B' and 'P'; Figure 2.1). Where a drug could be identified in two extracts, target *m/z* ions for that drug were included in both GC methods. Four *drugs.txt* files (Tables 2.6–2.9) were created which contained drug data used by the automated drug identification and quantitation macro to detect drugs (Chapter 8.).

It was necessary to minimise the number of target *m/z* ions monitored over any given time window during GC acquisition to ensure that a scanning rate of at least 3.0 cycles/s (Section 2.4.3.2) was obtained. Consequently, if a drug was able to be identified in more than two extracts (eg. carbamazepine, codeine, nordiazepam) target *m/z* ions were monitored in either:-
1. the two extracts for which drug recovery was greatest and least variable, or
2. in one underivatised and one derivatised extract rather than in the two underivatised extracts (Section 7.4).

7.4 Discussion

7.4.1 Drug Extraction Procedures

A comprehensive screen for over 100 pharmacologically and structurally diverse drugs in whole blood can be accomplished by:-

1. isolating target drugs from blood through an extraction procedure (combined LLE and SPE) where 90% of drugs are recovered with greater than 75% efficiency; and
2. identifying target drugs through the GC/MS analysis (SIM) of separate extracts in which drugs and their corresponding butyl or PFP derivatives are detected.

The LLE technique was based on a previously described method[23] but the use of a non-specific extraction solvent necessitated the incorporation of a 'cleanup' step prior to GC analysis. The price for 'cleaner' extracts, was a slight reduction in the recovery of some drugs. The reduction in drug recovery was an acceptable compromise given that the affected drugs were either well recovered by SPE (eg. benzodiazepines, THC-COOH) or were acidic drugs (eg, paracetamol, theophylline) which have comparatively high effective blood concentrations.

The SPE technique was based on a previously described method[32] but some immediate modifications were made as the unchanged method would fail to recover benzoylecgonine and morphine. It was essential that these drugs be recovered by the SPE technique as they were either not recovered (benzoylecgonine) or recovered poorly (morphine) by the LLE technique. Some of the published methods listed in Table 7.2 indicated the following:-

1. benzoylecgonine could be eluted with a more polar solvent (5% ammonium hydroxide in dichloromethane/isopropylalcohol 80:20); Method 20;([35])
2. opiates were well extracted in ammoniacal dichloromethane/isopropylalcohol (80:20); Method 20;[35]
3. excellent recoveries of benzoylecgonine, oxazepam and morphine with ammoniacal methanol; Method 14.[24]

The recovery of these drugs, however, with more polar eluants was at the expense of the cleanliness of the extract and additional endogenous matrix material was also extracted. Experiments in the present study indicate that, despite the use of polar eluants (Methods 'MEOH' and 'NH_3'), the chromatographic performance of the GC system was still satisfactory (Section 2.6.2) at the end of a sequence of GC analyses (although additional peaks were present in chromatograms). This was in contrast to the deterioration of the chromatographic performance observed following analysis of a sequence of liquid-liquid extracts. These observations confirm the literature[1] which found that SPE yields cleaner extracts then LLE and would, therefore, prolong the satisfactory chromatographic performance of the GC system (Section 9.4)

Apart from the presence or absence of a cholesterol and other blood-related GC peaks in chromatrograms, the absolute areas determined for temazepam and deuterated temazepam peaks were sensitive indicators of extract cleanliness. It is thought that the peak area of temazepam was reduced in the presence of co-extracted matrix products as the drug either adsorbed to these products in the injector or on the column, or adsorbed to active sites created in the injector in the presence of these products. Consequently, the chromatographic performance of temazepam and deuterated temazepam varied from sample to sample. The peak areas were greatest in solid-phase extracts, and smallest in Methods '8 + 21' and '8 + B21', presumably as a result of the accumulation of endogenous material in the final extract from the combined LLE methods.

The sum of SD (Table 7.6 - Note 4) associated with the recovery of all test drugs was greater following SPE than LLE. In Section 7.3.2 it was indicated that during the washing of the solid-phase packing and elution of drugs, the packing that was visible (packing immediately adjacent to the wall of the cartridge) appeared to be unevenly exposed to solutions passing through it. It is possible that within the packing some of the micro-channels between the particles of sorbent were partially or wholly occluded by matrix products which subsequently reduced the exposure of the sorbent particles to the eluants. Consequently, the reproducibility of extraction was compromised as indicated by the larger SD values obtained compared with LLE. It is also possible that the larger SD values may have arisen through experimental error as the SPE technique has a greater number of steps and is generally more manipulative than the LLE method.

It was consistently observed that the absolute peak area of deuterated THC-COOH was reduced following its analysis in blood extracts compared with that observed in B_{std}. As deuterated THC-COOH was added to extracts after LLE and SPE (Section 7.2.5), it underwent butylation in the presence of extracted blood products. It is thought these blood-related products, absent in B_{std}, interfered with the butylation of the drug. Consequently, the sensitivity of the instrument to THC-COOH has decreased (Section 9.4).

The unusual drug recovery data obtained for nifedipine, quinine and quinidine indicated that, although both LLE and SPE techniques could extract these drugs from blood, either their recovery from sample to sample, or chromatographic response from injection to injection, varied. In part, the variability in observed behaviour for derivatives of quinine and quinidine may have arisen due to their poor reproducibility and stability with time (Chapters 4 and 5). Consequently, if the drug screen were to identify one of these drugs, there would be no possibility of determining at what concentration it might be present in the blood.

7.4.2 Design of the Drug Screening Strategy

Data in Table 7.6 indicate that the LLE technique (Method 'AH') was most efficient in the extraction of weak to moderately acidic and basic drugs, and neutral drugs. The recovery of stronger acids (eg salicylic acid) and bases (eg. amphetamine) was relatively poor. Data in Table 7.6 indicate that the SPE technique (modified Method 17) was most efficient in the extraction of weakly acidic, neutral and weak to strongly basic drugs (including amphoteric drugs) but that the recovery of moderate to strong acids was poor. No one procedure was able to extract drugs with relatively high recoveries over the entire chemical range (strongly acidic to strongly basic).

If the broad drug screen employed both these extraction procedures (thus enabling detection of drugs over the entire chemical range), and if each of the 6 GC extracts was equivalent to 0.5 mL of blood (thus ensuring sufficient sensitivity; Table 6.1), then a total of 3 mL of blood would be required for the analysis. Total GC/MS analysis time would be long (4 hours with a 'turn around time' of 40 min from one GC injection to the next). Consequently, a drug screening strategy was devised (Figure 2.1) which minimised both the volume of blood required, and total GC/MS

analysis time, but which did not sacrifice the number and range of drugs identified. The drug screening strategy has the capacity to efficiently recover a chemically diverse range of drugs (moderately acidic to strongly basic drugs) and to detect strongly acidic drugs although their recovery is less efficient; however, their blood concentrations are usually high.

The analysis of a butylated extract produced following SPE is essential as this extract contains benzoylecgonine and THC-COOH (if present in the blood specimen). Analysis of this extract, however, does not exclusively identify any other target drug. Therefore, combining an extract from both LLE and SPE prior to butylation resulted in the following:-

1. a reduction in total GC/MS analysis time by approximately 40 min;
2. an increase in sensitivity towards some butyl drug derivatives; as the combined butyl extract is equivalent to 1 mL of blood, sensitivity will be increased where a drug is recovered by both LLE and SPE (eg. barbiturates, desipramine, diclofenac, desmethyl-benzodiazepine metabolites, phenytoin, warfarin).

The analysis of a pentafluoropropionylated extract produced following SPE is essential as this extract would contain the majority of drugs of (eg. amphetamine-like drugs, morphine, codeine, 7-amino-benzodiazepine metabolites, THC). Although some of these PFP derivatives would also be detected in the equivalent extract produced following LLE, analysis of the liquid-liquid extract does not exclusively identify any target drug. Consequently, a decision was made not to analyse a pentafluoropropionylated extract produced following LLE. This resulted in the following:-

1. a reduction in the total volume of blood required from 3 to 2.5 mL; and
2. a reduction in the total GC/MS analysis time by a further 40 min.

As a consequence, however, it was not possible to confirm the presence of some drugs by an alternate method (the 7-amino- benzodiazepine metabolites and some ß-blocking agents).

It was stated in Section 7.1 that, in the development of a drug extraction procedure, the aim was to meet as many of the seven requirements and six practical objectives as possible. One requirement was that the majority of drugs be identified and confirmed by at least two different methods. Through the drug screening strategy, confirmation of the identity of 60% of the target drugs is possible by an alternate method as follows:-

1. confirmation in an extract produced following extraction by a different technique (SPE or LLE), or
2. confirmation of a drug as its corresponding butyl or PFP derivative.

The identity of all drugs (except chloroquine, methaqualone, methylprylone and prednisolone) which would be detected in an underivatised extract could be confirmed in either the alternate underivatised extract ('L' or 'S'; Figure 2.1.), or as butyl or PFP derivatives. The identity of 47% of drugs detected as butyl derivatives and 48% of drugs detected as PFP derivatives could be confirmed in another extract. The drug screening strategy developed could be expected to satisfy the remaining requirements (Sections 1.3 and 1.4) and meet the practical objectives (Section 7.1)

although validation of the automated broad drug screening procedure would be necessary to determine the effectiveness of drug quantitation (Chapter 9).

References

1. "Comparison of liquid-liquid and solid-phase extraction techniques for drugs in urine and effects on the lifetime and performance of fused-silica capillary columns."
Rood D; Analytica Chimica Acta (1990) 236,115-120

2. "Preanalytical methodology for drug analysis in biological fluids."
Valentine J; Drug Fate Metab (1983) 4, 151-207

3. "Determination of 1,4-benzo(-diazepines) and (-diazepine-2-ones) on blood by ECD-GLC.
de Silva J; Anal Chem (1976) 48,1,10-19

4. "Sample preparation for organic acids in biological fluids."
Liebich H; Analytica Chimica Acta (1990) 236, 121-130

5. "Evaluation of the relative efficiency of various techniques for deproteinising plasma samples prior to HPLC analysis."
Blanchard J; J Chromatogr (1981) 226, 455-460

6. "Determination for some benzodiazepines and metabolites in serum, urine and saliva by HPLC."
Tjaden U; J Chromatogr (1980) 181, 227-241

7. "Sample preparation for biomedical analysis."
McDowall R; J Chromatogr Biomed Appl (1989) 492,3-58

8. "Fundamentals of drug metabolism and drug disposition."
LaDu B (Ed) 1971; Waverly Press Inc. Baltimore USA 419-423

9. "Trends in techniques for the extraction of drugs and pesticides from biological specimens prior to chromatographic separation and detection."
Furton K, Rein J; Analytica Chimica Acta (1990) 236,99-114

10. "Textbook of Practical Organic Chemistry."
Vogel (Ed) 1962; Longmans London.

11. "Isolation of drugs and drug metabolites from biological fluids by use of salt-solvent pairs."
Horning M; Clin Chem (1974) 20, 282-287

12. "A review of solid phase extractions: Basic principles and new developments."
Berrueta L, Gallo B, Vincente F: Chromatographia (1995) 40, 474-483

13. "Solid-phase extraction of drugs from biological tissues - A review."
Scheurer J, Moore C: J Anal Tox (1992) 16, 264-269

14. "Isolation of acidic, neutral and basic drugs form whole blood using a single mixed-mode solid-phase extraction column."
Chen X, Franke J, Wijsbeek J; J Anal Tox (1992) 16, 351-355

15. "Toxicological analysis of whole blood samples by means of Bond-Elut Certify columns and gas chromatography with nitrogen-phosphorus detection."
Zweipfenning P, Wilderink A, Horsthius P; J Chromatogr A (1994) 674, 87-95

16. "A new rapid gas chromatography method for the detection of basic drugs in postmortem blood, using a nitrogen phosphorus detector. Part I. Qualitative analysis."
Pierce W, Lamoreaux T, Urry F; J Anal Tox (1978) 2, 26-31

17. "Screening biological samples for underivatised drugs using a splitless injection technique on fused silica capillary column gas chromatography."
Ehresman D, Price S, Lakatua D; J Anal Tox (1985) 9,55-62

18. "An evaluation of fused silica capillary columns for the screening of basic drugs in postmortem blood: qualitative and quantitative analysis."
Koves E, Wells J; J Forensic Sci (1985) 30,692-707

19. "Testing for basic drugs in biological fluids by solvent extraction and dual capillary GC/NPD."
Fretthold D, Jones P, Sebrosky G; J Anal Tox (1986) 10, 10-14

20. "Screening of basic drugs in biological samples using dual column capillary chromatography and nitrogen-phosphorus detectors."
Watts V, Simonick T; J Anal Tox (1986) 10,198-204

21. "A rapid method for the identification of acidic, neutral and basic drugs in postmortem liver specimens by Toxi-Lab®."
Whitter P, Cary P; J Anal Tox (1986) 10,68-71

22. "Evaluation of a screening procedure for basic and neutral drugs: n-butyl chloride extraction and megabore capillary gas chromatography."
Sharp M; J Canadian Society of Forensic Science (1986) 19,83-101

23. "A rapid screening procedure for acidic and neutral drugs in blood by high resolution gas chromatography."
Sharp M; J Anal Tox (1987) 11, 811

24. "A simplified procedure for the isolation, characterisation , and identification of weak acid and neutral drugs from whole blood."
Anderson W, Fuller D; J Anal Tox (1987) 11, 198-204

25. "Screening for neutral and basic drugs in blood by dual fused-silica column chromatography with nitrogen-phosphorus detection."
Turcant A, Premel-Cabic A, Callieus A; Clin Chem (1988) 34, 1492-1497

26. "A single-step extraction for screening whole blood for basic drugs by capillary GC/NPD."
Cox R, Crifasi J, Dickey R; J Anal Tox (1989) 13,224-228

27. "Screening and confirmation of drugs in horse urine by using a simple column extraction procedure."
Singh A, Ashraf M, Granley U; J Chromatogr (1989) 473, 215-226

28. "Solid-phase extraction for the screening of acidic, neutral and basic drugs in plasma using a single-column procedure on Bond Elut Certify."
Chen X, Wisjbeek J, Van Veen J; J Chromatogr (1990) 529, 161-166

29. "Rapid screening for 100 basic drugs and metabolites in urine using cation exchange solid-phase extraction and high-performance liquid chromatography with diode array detection."
Logan B, Stafford D; J Anal Tox (1990) 14, 154-159

30. "Automated quantitative screening for acidic and neutral drugs in whole blood by dual-column capillary gas chromatography."
Ojanpera I, Rasanen I, Vuori E; J Anal Tox (1991) 15, 204-208

31. "Application of simple extraction procedure using aqueous ammonia to the analysis of basic drugs in blood by gas chromatography."
Sims D, Felgate H, Lokan R; Forensic Sci Int (1991) 49,33-42

32. "A single-column procedure on Bond Elut Certify for systematic toxicological analysis of drugs in plasma and urine."
Chen X, Wijsbeek J, Franke J; J Forensic Sci (1992) 37,61-71

33. "Screening of basic, neutral and weakly acidic drugs from post mortem blood using solid phase extraction procedure and GC."
Eklund A, Wikstrom M; TIAFT Proceedings of the 30th International Meeting (1992) 232-235

34. "Isolation of acidic, neutral and basic drugs from whole blood using a single mixed-mode solid-phase extraction column."
Chen X, Franke J, Wijsbeek J; J Anal Tox (1992) 16, 351-355

35. "Drug of abuse confirmation in human urine using stepwise solid-phase extraction and micellar electrokinetic capillary chromatography."
Wernly P, Thorman W; Anal Chem (1992) 2155-2159

36. "Capillary gas chromatographic drugs screen for use in forensic toxicology."
Drummer O, Horomidis S, Kourtis S; J Anal Tox (1994) 18, 134-138

37. "Toxicological analysis of whole blood samples by means of Bond-Elut Certify Columns and gas chromatography with nitrogen-phosphorus detection."
Zweipfenning P, Wilderink A, Horsthius P; J Chromatogr A (1994) 674, 87-95

38. "Determination of basic drugs extracted from biological matrices by means of solid-phase extraction and wider-bore capillary gas chromatography with nitrogen-phosphorus detection."
Chen X, Franke J Wijsbeek J; J Anal Tox (1994) 18, 150-153

Chapter 8.

AUTOMATED DRUG IDENTIFICATION AND QUANTITATION

8.1 Introduction

During discussion of the needs and limitations of a forensic laboratory and the development of an ideal drug screen (Sections 1.2 and 1.3) it was indicated that a screening procedure which automated the interpretation of complex multi-peak chromatograms and gave an indication of the concentration of drug present in the specimen would be highly advantageous.

It was indicated in Section 1.4.1 that automated identification of drugs in chromatograms (GC or HPLC) is currently based on matching either the RI or spectra (eg UV and mass spectra) of unknown peaks against commercially available or 'in-house' libraries of data on similar drugs. In the following studies, the RI of unknown peaks in complex chromatograms were automatically calculated and compared with a library of drug RI. The matches were identified as drugs.

In two studies[1,2] a drug RI was calculated through linear interpolation between adjacent HC markers. Each day a HC marker calibration standard was run independently of blood extracts (C_9-C_{26}, C_{28}, C_{30} and C_{32} ;and C_{10}-C_{34} except C_{33} respectively). The HC marker retention times obtained for that day were used to calculate the RI of all unknowns in the chromatogram. Peaks were identified as drugs when the calculated RI matched a drug RI in a library of over 100 drugs. Windows of ± 3.5 and ± 5 RI units were set respectively.

Another study[3] reported automated drug identification on the basis of drug RI calculated from a test mix of eleven drugs. In this case, the relationship between drug retention time and RI was not linear and, consequently, the slope and intercept of lines connecting adjacent drugs in the test mix were determined. The RI of an unknown was calculated from those of the two drug standards which bracketed it and was matched against a RI library of 112 drugs. A window of ± 10 RI units was set. A similar study[4] using a test mix of nine drugs obtained a linear relationship between retention time and molecular mass and, consequently, the RI of unknowns was determined via linear interpolation between 'bracketing' test drugs. A window of ± 5 RI units was set and the RI of unknowns matched with drug RI in a library of over 170 drugs.

The narrow windows of up to ± 10 RI units applied in these studies indicate the accuracy with which RI values can be assigned to GC peaks using a marker system (HC or drug).

Another study[5] reported automated identification and quantitation of 30 weakly acidic and neutral drugs. Identification was based on drug RI and determined through interpolation from nitrogen-containing standards fitted with a cubic spline (non-linear). Quantitation was based on a single point calibration (through zero) and

relative response to a single internal standard (which was also one of the standards used to determine drug RI). The quantitative precision of the method appeared good and was based on 10 separate determinations over 3 months, but was shown for only 8 drugs.

In Section 1.4.1 it was argued that drug identification based on only a single criterion (RI) was relatively non-specific (compared with other more specific techniques; eg GC/MS, HPLC-DAD) and the identity of the eluting peak required additional confirmation. An endogenous blood product, other contaminant or non-target drug with characteristics which cause it to elute with the same RI as a drug may be falsely identified as that drug. Similarly, should conditions arise which cause the RI of an eluting drug to shift slightly (Appendix A.4) then it may not be matched with the corresponding drug standard and a false negative would result. For example, the RI of acidic, neutral and basic drugs showed concentration-dependent behaviour with marked increases in RI when concentrations exceeded 100 µg/mL. In some cases, the RI differences caused by differing concentration were over 100 RI units[6]. This phenomenon was also observed in the present study (Section 8.4.2).

More specific drug identification would be obtained through mass spectrometry. The advantages and disadvantages of full-scan and SIM mass spectrometry were discussed in Section 1.4.2. Searching complex, multi-peak chromatograms generated by mass spectrometry for drugs is generally performed either:-

1. automatically by commercial or 'in-house' full-scan library search programs,[7] or
2. manually by the experienced analyst.

The advantages and disadvantages of each method were discussed in Section 1.2.

Some studies [8-12]employing GC/MS report more specific and sensitive automated drug identification. For example, screening for specific drug groups and/or their metabolites was achieved by monitoring (SIM) groups of typical fragment *m/z* ions which, in combination, uniquely identified the drugs from other co-chromatographing compounds. Following data acquisition, the *m/z* ions which identified a specific drug from the group were automatically reconstructed in a 'mass fragmentogram' which was compared with a library full-scan mass spectrum of the drug. Individual drugs from target analyses for benzodiazepines[8,9], ß-blocking agents[10], neuroleptics[11] and anti-inflammatory agents[12] have been automatically identified in urine extracts employing this technique. Disadvantages associated with this approach to target drug analyses, however, are as follows:-

1. the *m/z* ions monitored were generally characteristic of acetylated products rather than the drug itself, and
2. the technique could not be applied to the identification of chemically dissimilar drugs in broad drug screening.

In addition, the application was developed in urine rather than blood.

One study[13] described automated identification of a diverse range of drugs in SIM generated ion chromatograms from blood extracts. A pair of target *m/z* ions which characterised each drug or drug derivative was monitored over a narrow time window about the expected retention time of the drug. A peak was identified as a drug if both the target *m/z* ions and their relative *m/z* ion abundance matched those of reference

standards. The macro which automated drug identification in multi-peak chromatograms consisted of repeating units. These units contained the drug name, retention time, the *m/z* target ions, integration threshold and the permissible range of ion ratio. In contrast with other automated drug identification techniques, this procedure utilised both retention time and specific mass spectral information in the identification of a diverse range of drugs providing both specificity and sensitivity. However, the application of the macro was restricted to the instrument on which it was developed as, for each target drug, it was dependent on absolute retention time (governed by GC parameters), absolute peak area threshold (instrument specific) and on relative ion abundance (dependent on MS tuning parameters). Similarly, the macro was developed on the now relatively obsolete Hewlett Packard 59970A Chemstation with Pascal software.

The automated drug identification and quantitation technique developed in the present work was an extension of that used in the study above.[13] It was designed for universal application to complex chromatograms generated on any non-polar column and any mass spectrometer and automates both drug identification and quantitation.

8.2 Methods

Chromatograms generated from GC/MS analyses of blood extracts and underivatised and derivatised drug solutions were used in the development of the macro.

Refer to "General Procedure" (Chapter 2). In particular, for HC marker solution refer to Section 2.2.5.5 and 2.2.5.6; for reference drug solutions refer to Section 2.2.5.14; For analyses with the Hewlett Packard GC/MSD refer to Section 2.4.1 for instrumentation; Sections 2.4.3.2 and 2.6.2 for SIM acquisition parameters; Section 2.4.2 or gas chromatographic parameters with the wider bore column employing splitless injection; and Section 2.5 for drug extraction from blood and derivatisation techniques.

8.3 Results

8.3.1 The Macro

Automated drug detection and quantitation was performed by a series of eight macros which themselves used internal macros and commands recognised by the Hewlett Packard (HP) operating software. 'Hits' identified by the macro are listed in an automatically generated report which displays the *m/z* ion chromatograms for each 'hit' together with other relevant information (Section 8.3.2). The analyst interprets each macro-generated report and eliminates false 'hits' identified by the macro (Section 8.3.3).

The data processing of all blood extracts was performed by identical macros which differed only in the text generated for the final report. The following series of macros were used for the detection of butyl drug derivatives and consequently, the text generated relates specifically to butyl extracts. The macros employ internal macros and commands recognised by Hewlett Packard operating software (eg.

Ret_Time, Peak_Area, rteintparams, peakvars, TIC, print using #). Each new macro is preceded by 'NAME' and comments (in *italics*) which briefly describe the working of the macro appear throughout the program. During the development of the 'CheckFor' macro, 'debugging' messages were used to help locate the point at which the macro failed. These debugging messages appear in a smaller font and do not routinely operate unless activated by the user at the command line or in the 'CustomAnalysis' macro.

Start automated drug detection and quantitation macro.

```
NAME Locate Hydrocarbons
```

A macro which locates HC marker GC peaks and records their retention times (RTs).

```
local RI,start,finish
orientpr = 1
print #2,
"                                                                                "
print "Locating markers"
print #2,""
print #2,"Peaks identified as HC marker markers"
print #2,""
```

Macro chromatographs m/z 71 (the HC marker m/z ion which is monitored during data acquisition) and integrates the largest 20 peaks; the integrated chromatogram is stored in the R1 register.

```
rteintparms 0,0,20,1,0.2,0.0,5,100,0,0,1,0,
chrom ,71
rteint x
LastPeak = npeaks
draw
R1=X
```

Macro locates the HC marker peaks by assuming that the last three peaks in the integrated chromatogram are C_{32}, C_{34} and C_{36} (refer 8.4.1); RTs and Peak Areas (PAs) are stored in two individual arrays of 14 for future reference.

last integrated peak = C_{36}

```
PEAKNUMBER LastPeak,TOP,R1
RTs[14] = Ret_Time
print #2,"Marker   Time      Area"
print using #2,"C##   ##.###   ########/",36,Ret_Time,Peak_Area
PAs[14] = Peak_Area
```

second-last peak = C_{34}

```
PEAKNUMBER LastPeak-1,TOP,R1
RTs[13] = Ret_Time
print using #2,"C##   ##.###   ########/",34,Ret_Time,Peak_Area
PAs[13] = Peak_Area
```

third-last peak = C_{32}

```
PEAKNUMBER LastPeak-2,TOP,R1
RTs[12] = Ret_Time
print using #2,"C##   ##.###   ########/",32,Ret_Time,Peak_Area
PAs[12] = Peak_Area
```

Macro sets the initial interval to the next HC marker (C_{30}) as the RT difference between C_{32} and C_{34},

```
interval = RTs[13] - RTs[12]
```

and works backwards looking for HC markers; the interval is adjusted to that between the last found HC markers ± 0.5 min to better locate the next expected HC marker in the series.

```
last=12
i=11
n = 11
rteintparms ,,1

while i > 0 do
NumToRI i,RI
  expect = RTs[last] + ((i-last)*interval)
  start = expect - 0.5
  finish = expect + 0.5
  if finish > File_Start then
    print "Looking for C"+val$(RI/100)
    if start < File_Start then
      start = File_Start
    endif
    chrom start:finish,71
    rteint
    draw 1
    if npeaks = 1 then
```

Macro found the next expected HC marker; RT and PA for that HC marker are placed into the arrays.

```
      PEAKNUMBER 1,TOP,X
      RTs[i] = Ret_Time
      PAs[n] = Peak_Area
      print using #2,"C##   ##.###   ########/",RI/100,Ret_Time,Peak_Area
```

Macro sets new interval

```
      interval = (RTs[last] - RTs[i]) / (last - i)
      last = i
  i = i - 1
  n = n - 1
endwhile
```

The Area Threshold for peak detection is set to a fraction of C_{32} peak area such that no absolute threshold value needs to be set (Section 8.4.2).

AreaThreshold = Pas[12]/10000

```
print #2,""
print #2,"Area threshold set to "+val$(AreaThreshold)
print #2,""
return
```

End of macro 'LocateHydrocarbons'.

NAME CheckFor

A macro which checks for a target drug on the basis of Retenion Index (RIndex) and a pair of identifying m/z ions (mass1 and mass 2) which must have coincident RTs; parameters for each drug are listed in a table - drugs.txt (Section 2.7).

```
parameter name$,Rindex,mass1,mass2
local rtime,ActualRI
print "Check for " + name$
  if debugging then
    print #2,"Check for "+name$
  endif
```

Macro converts drug RIndex listed in drugs.txt to a corresponding retention time (rtime) on the basis of the RTs determined for the HC markers found in the current chromatogram; the conversion is performed by the 'RIToTime' macro.

```
RIToTime rindex,rtime
```

The two m/z ions for each drug are chromatographed over a time window (± 0.2 min) around the calculated rtime.

```
delta = 0.20
chrom rtime-delta:rtime+delta,mass1
rteintparms 0,0,1,-1,0.2,0.0,5,100,0,0,1,0,
rteint x
peakvars 1,top,x
area1 = Peak_Area
time1 = Ret_Time
```

If mass1 is found, the peak area and retention time are assigned as variables area1 and time1 respectively.

If no mass1 is found then the drug is not identified/detected by the macro.

```
if npeaks = 0 then
  if debugging then
   print #2,name$ + " " val$(rtime) + " " + val$(mass1) + " missing"
   endif
  return
endif

chrom rtime-delta:rtime+delta,mass2
```

Macro stores the mass2 chromatogram for later use in the R1 register.

```
R1=X
```

Up to two peaks are integrated in the ion chromatogram for mass2 and the peak height of the tallest peak is assigned a variable (Ytop) for later use.

```
rteintparms ,,2,-1
rteint x
getscalars ,,x
Ytop=Yhigh
```

If no mass2 is found then the drug is not identified/detected by the macro..

```
if npeaks = 0 then
  if debugging then
    print #2,name$ + " " + val$(rtime) + " " + val$(mass2) + " missing"
  endif
  return
endif
```

If only one mass2 peak is found in the time window, then the PA and RT are assigned as variables area2 and time2 respectively.

```
if npeaks = 1 then
 peakvars 1,top,x
 area2 = Peak_Area
 time2 = Ret_Time
endif
```

The macro checks that the RTs of mass1 and mass2 are coincident to within 0.03 min; if RTs are not coincident (> 0.03 min) then no drug is identified/detected by the macro.

```
if (npeaks =1 and abs(time1 - time2) > 0.03) then
  if debugging then
   print #2,name$ + " peak times too far apart " + val$(time1) + " " + val$(time2)
  endif
  return
endif
```

If mass1 and mass2 peaks are coincident then the PA and RT of mass2 are assigned as variables used later in quantitation (areaq and timeq respectively).

```
if (npeaks =1 and abs(time1 - time2) < 0.03) then
 timeq = time2
 areaq = area2
endif
```

If two peaks were integrated in the mass2 ion chromatogram then the peak that is coincident (≤ 0.03 min) with mass1 is determined (Section 8.4.2).

```
if npeaks = 2 then
 peakvars 1,top,x
 area3 = Peak_Area
 time3 = Ret_Time
 peakvars 2,top,x
 area4 = Peak_Area
 time4 = Ret_Time
endif

if (npeaks = 2 and (abs(time1 - time3)> 0.03) and (abs(time1 - time4) > 0.03)) then
  if debugging then
   print #2,name$ + " peak times too far apart " + val$(time1) + " " + val$(time2)
  endif
  return
endif
```

The PA and RT of the appropriate mass2 peak are assigned as variables used later in quantitation (areaq and timeq respectively).

```
if (npeaks = 2 and abs(time1 - time3) < 0.03) then
 timeq = time3
 areaq = area3
endif
if (npeaks = 2 and abs(time1 - time4) < 0.03) then
 timeq = time4
 areaq = area4
endif
```

The average RT of mass1 and mass2 is recorded as the RT of the drug detected,

```
AverageTime = (time1 + timeq)/2
```

and is rounded to two decimal places for the report.

```
AverageT = (trunc (((AverageTime*1000) + 5)/10))/100
```

The RT of the drug is calculated as a RIndex (performed by the macro 'TimeToRI'), compared with the RIndex listed for that drug in drugs.txt, and the retention index error (RI error) calculated (rounded to the nearest whole number for the report).

```
TimeToRI AverageTime,ActualRI
RIDifference = RIndex - ActualRI
if RIndex > ActualRI then
 RIDiff = trunc(((RIDifference *10)+5)/10)
endif
if Acutal RI > Rindex then
 RIDiff = trunc(((RIDifference *10)-5)/10)
endif
```

Drugs detected by the macro are not reported as 'hits' if any one of the following three statements is satisfied:-

1). If the RI error determined is too great (> ± 25);Section 8.4.2

```
if abs(RIDifference) > 25 then
  if debugging then
   print #2,name$ + " retention index difference greater than 20 " +RIDifference
  endif
  return
endif
```

2). If the PA of mass2 is less than 0.001 of the PA of mass1; Section 8.4.2.

```
if ((area1 > AreaThreshold) and (areaq < area1/1000)) then
return
endif
```

3). If the PA of mass1 is less than the AreaThreshold; Section 8.4.2.

```
if ((area1 < AreaThreshold) and (areaq > area1/1000)) then
return
endif
```

If none of the statements is satisifed, the drug detected is recorded as a 'hit' and placed in an array.

```
if ((area1 > AreaThreshold) and (areaq > area1/1000)) then
z=1
  hits$ = ""
hits = hits + 1
  found[hits] = AverageTime
```

The macro determines whether the current 'hit' is coincident with another and reports the other coincident 'hit' (identified by a number #x); Section 8.4.2.

```
    while z < hits do
      if abs(AverageTime - found[z]) < 0.03 then
coincident
      hits$ = "=#" + val$(z)
      z = hits
     endif
    z = z + 1
    endwhile
```

The macro determines whether the current 'hit' is coincident with a HC marker and reports the HC marker with which it is coincident; Section 8.4.2.

```
X = 1
while X < 15 do
 if RTs[X] > 0 and abs(AverageTime - RTs[X]) < 0.03 then
coincident
   if not eqstr(hits$, "")
    hits$ = hits$ + ","
   endif
   hits$ = hits$ + "=C" + val$(X*2 + 8)
  endif
X = X + 1
endwhile
```

The macro determines whether the RI error of current 'hit' is larger than expected (10< RIerror > 25) and reports an "Analyst Alert" - ''.*

```
errors$ = ""
if abs(RIDifference) > 10 then
  if not eqstr(errors$, "") then
   errors$ = errors$ + ","
  endif
  errors$ = errors$ + "*"
endif
```

The macro re-integrates mass1 with integration parameters that allow for either tailing or nosiy peak profiles and assigns the new PA of mass1 as a variable (areaT) used in quantitation (Section 8.4.2).

```
chrom AverageTime-(delta/2):AverageTime+(delta*2),mass1
rteintparms 0,0.0,2,-1,0.2,0.4,0,35,0,1,1,0
rteint x
```

The peak height of the peak is assigned a variable (Yhigh) and is compared with Ytop determined for the mass2 peak. Which ever is the tallest peak is assigned Ytop for later use.

```
get scalars point ,,x
if Yhigh > Ytop then
 Ytop=Yhigh
endif
if npeaks = 1 then
 peakvars 1,top,x
 areaT = Peak_Area
endif
```

By broadening the time window over which the mass1 m/z ion is integrated, a second peak with incorrect RT but larger PA than the target peak may be integrated and potentially used in quantitation by error; therefore the macro determines that the correct peak was within 0.03 min of AverageTime (drug RT).

```
if npeaks = 2 then
 peakvars 1,top,x
  area5 = Peak_Area
  time5 = Ret_Time
 peakvars 2,top,x
  area6 = Peak_Area
  time6 = Ret_Time
endif

if (npeaks = 2 and abs(AverageTime - time5) < 0.03) then
 areaT = area5
endif
if (npeaks = 2 and abs(AverageTime - time6) < 0.03) then
 areaT = area6
endif
```

In the event that the wrong peak was integrated as a result of broadening the time window (AverageTime - RT > 0.03) an "Analyst Alert" - '¤' is reported.

```
quant$ = ""
if (npeaks = 2 and (abs(AverageTime - time5) > 0.03) and
(abs(AverageTime - time6) > 0.03)) then
  if not eqstr(quant$, "") then
   quant$ = quant$ + ","
  endif
  quant$ = quant$ + "¤"
endif
```

Calculation of quantitative value; Section 8.3.3.2.

```
PAn=PAs[val(n$)]
logquant=(log(areaT/(PAn*2))+val(C$))/val(M$)
quant = EXP(logquant*ln(10))*100/val(Recovery$)
quant1 = (trunc(((quant*10000) +%)/10))/1000
```

```
if not eqstr(hits$, "") then
  hits$ = "(" + hits$ + ")"
endif
```

If no calibration curve was determined for this drug (no M and C values) then the macro reports the blood level as '99.999'.

```
if val(C$) = 9 and val(M$) = 9 then
quant1 = 99.999
endif
```

If no recovery from blood data was determined for this drug then the macro reports an "Analyst Alert" (<) which indicates that the minimum blood level has been reported.

```
if val(Recovery$) = 100 then
 if not eqstr(quant$, "") then
   quant$ = quant$ + ","
 endif
 quant$ = quant$ + "<"
endif
```

If recovery from blood was found to be very variable for this drug then the macro reports an "Analyst Alert" - '±'.

```
if val(Recovery$) = 200 then
 if not eqstr(quant$, "") then
   quant$ = quant$ + ","
 endif
 quant$ = quant$ + "±"
endif
```

The macro displays both m/z ions for the current 'hit' in a specified window and prints the window to the report together with related information (name, RT, RIerror, absolute mass1 peak area, blood concentration, extract in which to confirm the presence of the drug). The position on the page is specified for each 'hit' (Section 8.4.2).

```
Y=R1
mer
format merged
w = w + 1

if w = 1 then
 startprint
endif

 draw (w),x,AverageTime-(delta/2):AverageTime+(delta*2.5), 0:Ytop*1.3
 strprint "#" + val$(hits) + ": " + name$,50,w*10-6
 strprint "Ret.Time:    " + val$(AverageTime) + hits$,50,w*10-5
 strprint "RI error:    " + val$(RIDifference) + errors$,50,w*10-4
 strprint "Mass 1 area: " + val$(areaT),50,w*10-3
```

```
    strprint "Conc(µg/mL): " + val$(quant1) + quant$,50,w*10-2
    strprint "confirm in:  " + confirm$,50,w*10-1
    winprint w,4,w*10-6,45,10

  if w = 1 then
   R2=x
  endif
  if w = 2 then
   R3 = x
  endif
  if w = 3 then
   R4 = x
  endif
  if w = 4 then
   R5 = x
  endif
  if w = 5 then
   R6 = x
  endif
  if w = 6 then
   R7 = x
```

The macro prints a page which displays the chromatogram and related information for each of 6 'hits' found. The windows are then cleared and filled sequentially again until a further 6 'hits' are accumulated, and another page is printed.

```
  endprint
  w=0
  clear
  endif

  endif
  return
```

End of macro 'CheckFor'

NAME TimeToRI

A macro used in the 'CheckFor' macro which converts the RT determined for a detected drug to a RI. The RT to RI conversion is based on linear interpolation between the two HC markers which bracket the drug and for which a RT and RI are known (stored in arrays).

```
  parameter RT,RI by value
  local ri1,ri2,n1,n2,t1,t2
```

The macro locates array index containing earliest time AFTER drug RT

```
  n2 = 1
  while RTs[n2] < RT and n2 < 14
    n2 = n2 + 1
  endwhile
  t2 = RTs[n2]
```

```
The macro locates array index with latest time BEFORE drug RT
   n1 = n2-1

   if n2 = 0 then
      return
   endif

   while RTs[n1] = 0 and n1 > 1
      n1 = n1 - 1
   endwhile
   t1 = RTs[n1]

   NumToRI n1,ri1
   NumToRI n2,ri2

   if n1 = n2 then
      return
   endif

   RI = ri1 + (ri2-ri1)*(RT-t1)/(t2-t1)
   return
End macro TimeToRI
```

NAME RIToTime

A macro used in the 'CheckFor' macro to convert the RI listed in drugs.txt to a RT for each drug. The RI to RT conversion is based on linear interpolation between the two HC markers which bracket the drug and for which a RT and RI are known (stored in arrays).

```
   parameter RIndex, rtime by value
   local n1,n2,rt1,rt2,ri1,ri2

   RIToNum RIndex,n1
   n2 = n1 + 1
   while RTs[n1] = 0 and n1 > 1 do
      n1 = n1 - 1
   endwhile
   while RTs[n2] = 0 and n2 < 14 do
      n2 = n2 + 1
   endwhile
   NumToRI n1,ri1
   rt1 = RTs[n1]
   NumToRI n2,ri2
   rt2 = RTs[n2]
   rtime = rt1 + (RIndex-ri1)*(rt2-rt1)/(ri2-ri1)
   return
End of macro 'RIToTime'
```

NAME PickOff

A macro which allows the 'CheckFor' macro to refer to and use the different parameters listed in drugs.txt which describe each target drug.

```
parameter s by value
p = instr(line$,",")
if p = 0 then
  s = line$
  line$ = ""
else
  s = line$[1:p-1]
  line$ = line$[p+1:len(line$)]
endif
return
```

End of macro 'PickOff'

NAME RIToNum

A macro which converts the retention index numbers for the HC markers (1000, 1200, 1400 etc) to array numbers (1, 2, 3 etc) and is used in the 'LocateHydrocarbons', 'RIToTime' and 'TimeToRI' macros.

```
parameter RI, n by value
n = trunc ((RI-1000)/200) + 1
return
```

End of macro 'RIToNum'

NAME NumToRI

A macro which converts the array numbers (1, 2, 3 etc) for the HC markers to retention index numbers (1000, 1200, 1400 etc) and used in the 'LocateHydrocarbons', 'RIToTime' and 'TimeToRI' macros.

```
parameter n, RI by value
RI = 1000 + (200*(n-1))
return
```

End of macro 'NumToRI'

NAME CustomAnalysis

The 'top level' macro which directs the automated drug search and prints the final report.

Turns 'debugging' on or off

```
if CHECK("VARIABLE","debugging") = 0 then
  debugging = 0
endif
```

The following are specific lines of text related to report generation for butyl extracts.

```
footer$ = "**Combined SPE and LLE**          "
pagenum = 1
```

```
path$ = _MethPath$ + _MethFile$ + "/"
open path$ + "drugs.txt" for input as #1
open path$ + "REPORT.TXT" for output as #2
print #2, ""
print #2, "          Broad Drug Screen in Blood for Drugs "
print #2, "               (Butyl Derivatives)"
print #2, ""
print #2, ""
print #2,"Data File : " + _DataFile$
print #2,"Data Name : " + _DataName$
print #2,"Misc Info : " + _MiscInfo$
```

The macro creates arrays for HC marker RTs and PAs (up to 14) and 'hits' (up to 60).

```
dim RTs,14
dim PAs,14
dim found,60
hits = 0
w = 0
```

Some arbitrary values assigned to variables used by the 'CheckFor' macro; Section 8.4.2

```
time2=200
time3=300
time4=400
time5=500
time6=600
```

Initiates the 'LocateHydrocarbons' macro.

```
LocateHydrocarbons
```

The macro continues to refer to drugs.txt for target drugs and their relevant parameters until it reaches the line "--END--".

```
input #1,line$
while not eqstr(line$,"--END--") do
drug$ = ""
  ri$ = ""
  m1$ = ""
  m2$ = ""
  n$ = ""
  confirm$ = ""
  C$ = ""
  M$ =""
  Recovery$ = ""
```

The macro ignores any lines in drugs.txt preceded by "".*

```
  if not eqstr(line$[1:1], "*") then
    PickOff drug$
    PickOff ri$
```

```
        PickOff m1$
        PickOff m2$
        PickOff n$
        PickOff confirm$
        PickOff C$
        PickOff M$
        PickOff Recovery$
        ri = val(ri$)
        m1 = val(m1$)
        m2 = val(m2$)
        n = val(n$)
        confirm = val(confirm$)
        C = val(C$)
        M = val(M$)
        Recovery = val(Recovery$)
```

Initiates 'CheckFor' macro.

```
        CheckFor drug$,ri,m1,m2
      endif
      input #1,line$
   endwhile
   close #1
```

In the event that no 'hits' are found, the macro reports 'No Drugs Detected'.

```
   if hits = 0 then
   print    #2,"   ** NO DRUGS DETECTED**"
   endif
```

With the completion of the 'CheckFor' macro, the final report is generated which lists the various notes and "Analyst Alerts" relevant to the 'hits'.

```
   print
   #2,"_____________________________________________________________"
   print #2,""
   print #2,"   Analyst Alerts"
   print #2,""
   print #2,"   *     = Absolute RIerror > 10."
   print #2,"   ¤     = Quantitation incorrect!  The wrong peak was integrated by "
   print #2,"           the macro - refer to chromatogram for that drug."
   print #2,"   <     = No recovery data.  Report 'at least' x µg/mL."
   print #2,"   ±     = Recovery from blood very variable."
   print #2,""
   print #2,"   Notes"
   print #2,""
   print #2,"   ¹          = Area of drug target ion > set threshold area."
   print #2,"   ²          = Macro calculated RI - Actual RI."
   print #2,"   99.999 = No quantitative value due to poor reproduciblity of either"
   print #2,"                the day-to-day GC reponse, or derivatization."
   print #2,"   LLE    = Liquid-liquid extract."
```

```
print #2,"   SPE   = Solid phase extract."
print #2,"   PFP   = Pentafluoropropionylated solid phase extract."
!print #2,"  BUTYL  = Butylated extract (combined LLE and SPE)."
print #2,""
print #2,""
print #2,""
print     #2,"              **ANALYST INTERPRETATION**"
print     #2,""
print #2,"                                                    µg/mL"
print     #2,"              DETECTED DRUGS     1. "
print     #2,"                                 2. "
print     #2,"                                 3."
print     #2,"                                 4."
print     #2,"                                 5."
print     #2,"                                 6."
print #2,""
print #2,""
print #2,""
print     #2,"                                 _____"
print     #2,"                                   (Analyst)"
End report
   close #2
```

The macro prints the remaining windows (< window 6) generated in the 'CheckFor' macro.

```
   if w > 0 then
    endprint
    w=0
   clear
   endif
```

The macro prints the front page of the report.

```
   printtextfile "report.txt"
```

The macro prints the full page chromatogram generated by the butyl extract.

```
   orientpr = 2
   TIC
   dr 3,x
   startprint
   winprint 3,4,10,101,33
   endprint
   return
```

End automated drug detection and quantitation macro.

8.3.2 The Final Report

Report 8.1 is a reproduction of one page of a macro-generated report following the GC/MS analysis of a derivatised (PFPA) mixed drug solution. The drug solution contained 19 drugs at concentrations of approximately 1 µg/mL. The first six

'hits' identified by the macro are displayed together with:- the retention time (Ret.Time); RI error; the absolute peak area of mass 1; the calculated drug concentration; and the alternate extract in which the same 'hit' would also be expected if the drug were present in the blood specimen. Of the six 'hits' shown in Report 8.1, the presence of only two can be confirmed in an alternate extract (carbamazepine and codeine). Common to all the 'hits' displayed in the report are the following.

1. 'Hits' are reported alphabetically.
2. 'Hits' are aligned and shown in the first third of the window.
3. The pair of target *m/z* ions which characterise each 'hit' are coincident.
4. Mass1 is the second *m/z* ion listed in each window (eg. for 7-amino flunitrazepam; mass1 = 401.00). Mass 1 is integrated (for quantitation) and an integration line is apparent.
5. The pair of target *m/z* ions have differing relative abundances but identical peak profiles. For example, alprenolol mass2 is much smaller than mass1; mass1 and mass2 of 7-aminoflunitrazepam are both moderately tailing.
6. Although each 'hit' is displayed so as to fill the window, the peaks shown have differing peak heights as indicated by abundance (*y* axis).
7. The RI error indicates that all 'hits' are within 10 RI units of the RI listed in *drugs.txt*.
8. The calculated drug concentration approximates the true concentration of the mixed drug solution.

Reports 8.2–8.5 are reproductions of the reports generated following the analysis for drugs of a single forensic blood specimen (Figure 2.1). Reports 8.2 and 8.3 were generated from underivatised blood extracts following liquid-liquid and solid-phase extractions respectively. Report 8.4 was generated from the butylated blood extract (combined fractions from liquid-liquid and solid phase extractions). Report 8.5 was generated from the pentafluoropropionylated blood extract following solid-phase extraction. The "Analyst Interpretation" (first page of each report) shows a list of "Drugs Detected". These are 'hits' identified by the macro which, following analyst interpretation, have been confirmed as drugs identified in each extract. Note that during macro development, typographical errors occurred while entering drug names into the *drugs.txt* file. These errors are permanently recorded in the macro reports and some are apparent in Reports 8.2–8.5.

8.3.3. Analyst Interpretation of Macro Generated Reports

8.3.3.1 *Elimination of False Positives*

Table 8.1 shows the completed summary sheet routinely used by the analyst to collate the drugs detected from each of the four reports generated following the analysis of a blood specimen. In this case, the four columns of data (left to right) correspond to the drugs detected in Reports 8.2–8.5 respectively. As indicated in Section 8.3.2, each macro-generated report indicates in which alternate extract a 'hit' might also be expected (eg. "confirm in: LLE/PFP"). Where the expected confirmation of a 'hit' from one extract did not occur in the other, the analyst indicates this with "- UNCONFIRMED -" (codeine, cotinine, trimethoprim). For all other drugs listed, there was either confirmation as expected (eg. amitriptyline, diazepam, methadone), or no alternate extract in which to confirm the presence of

a 'hit' (eg. paracetamol, 7-amino nitrazepam). The estimated blood concentration of the drug is given for each extract in which it was detected. No quantitation was performed by the macro for some drugs (-).

Based on the sequence of analyst checks outlined in Section 8.4.3, 'hits' identified in Reports 8.2–8.5 which possessed the necessary features to assign them as drugs were reported as 'Drugs Detected' by the analyst as follows:- amitriptyline and nortriptyline (metabolite), caffeine, carbamazepine, cotinine, diazepam and nordiazepam (metabolite), methadone, and paracetamol. The majority of 'hits' listed in the four reports had been falsely identified as drugs by the macro and were eliminated by the analyst for at least one of the nine reasons listed in Table 8.2. The false 'hits' from Reports 8.2–8.5 are listed below with the reason/s (1–9, Table 8.2) that the analyst did not include them in "Drugs Detected" lists.

Report 8.2 indicates the following drugs were present in the blood extract:- amitriptyline, caffeine, diazepam, methadone, nordiazepam.

Falsely identified 'hits' and reasons for their rejection (Table 8.2) are as follows:-

benzhexol (7)
chlorpheniramine (4,7)
codeine (7)
diphenhydramine (7)
methylphenidate (7)
metoclopramide (1,5)
moclobemide (1)
pentobarbitone (2,3,7)
pheniramine (1)
procainamide (1)
propoxyphene (1,2,3)
thioridazine (3,4,8)
trimipramine (4,7)
benztropine (8)
clomipramine (1,7)
dihydrocodeine (3,7)
fenfluramine (8)
methylphenobarbitone (7)
mianserin (2,7)
nifedipine (7)
phencyclidine (7)
phenylbutazone (7)
promethazine (7,8)
quinine (4,5)
tiaprofenic acid (7)

Report 8.3 indicates the following drugs were present in the blood extract:- amitriptyline; caffeine (relatively large and distorted peak); carbamazepine (identical tailing peak profiles for the two target *m/z* ions); codeine (although the analyst would expect a slightly poorer peak shape); cotinine (identical tailing peak profiles for the two target *m/z* ions); diazepam; methadone; and trimethoprim (grossly overloaded peak and consequently *m/z* ion abundances are meaningless).

Falsely identified 'hits' and reasons for their rejection (Table 8.2) are as follows:-

chloroquine (1,4)
diethylpropion (7)
diltiazem (7)
lignocaine (3,7)
methylphenidate (2,7)
moclobemide (1)
oxycodone (7)
phenindione (4,6,7)
chlorpheniramine (4)
dihydrocodeine (2,4)
diphenhydramine (7)
methaqualone (4,6,7)
methyprylone (2,3,6)
nifedipine (2,3,6)
pentobarbitone (7)
phenobarbitone (3,4,6)

phenylbutazone (2,6,7)
procainamide (3,7)
prochlorperazine (7)
quinine (3,7)
trimeprazine (7)

Report 8.4 correctly indicates the following drug derivatives were present in the butylated blood extract:- nordiazepam, nortriptyline, paracetamol.

Falsely identified 'hits' and reasons for their rejection (Table 8.2) are as follows:-

diclofenac (7)
diflunisal (7)
frusemide (7)
ibuprofen (7)
indomethacin (3,9)
methylphenobarbitone (4,7)
naproxen (7)
nitrazepam (1)
phenobarbitone (7)
phenytoin (7)
quinine (4,3)
sulindac (7,8)
theophylline (3,9)
tolbutamide (4,7)

Report 8.5 correctly indicates the following drug derivatives were present in the pentafluoropropionylated blood extract:- 7-aminonitrazepam, carbamazepine and nortriptyline.

Falsely identified 'hits' and reasons for their rejection (Table 8.2) were as follows:-

7-aminoflunitrazepam(7)
codeine (4)
desipramine (3,7)
ephedrine (4,7,9)
fenfluramine (3,7)
flecainide (7)
hyoscyamine (3)
MDMA (9)
metoclopramide (4,6)
oxyphenbutazone (3,7)
phenylpropanolamine (2,4,7)
pseudoephedrine (3,7,9)
sotalol (3,7)
warfarin (7)

For all drugs listed except 7-amino nitrazepam and paracetamol, a drug detected in one extract could be checked for in another, thus confirming the presence of that drug in the blood sample. The presence in the blood sample of amitriptyline, caffeine, carbamazepine, diazepam, methadone, nordiazepam and nortriptyline could all be confirmed in this way. Similarly, the presence of the drug metabolites, nordiazepam and nortriptyline, provided additional confirmation of the presence of the parent drugs, amitriptyline and diazepam, respectively.

In contrast, Table 8.1 indicates that trimethoprim, cotinine and codeine detected in the solid-phase blood extract (Report 8.3) were not confirmed in other extracts where their presence would have been expected ('- UNCONFIRMED -').

8.3.3.2. *Quantitation of Drugs Detected*

The 'CheckFor' macro estimates the concentration in blood of each 'hit' it identifies in a GC extract from a predetermined calibration curve based on log transformed data points (Section 3.3.1). The peak area ratio (Section 2.6.1) for each 'hit' identified by the macro is automatically calculated using the HC marker specified in *drugs.txt*. The C and M values which describe the linear regression equations fitted to log transformed data points are listed in the *drugs.txt*. file to which the 'CheckFor' macro

refers. The C and M values were determined for each drug from reference drug solutions at concentrations of approximately 0.25 μg/mL up to approximately 3 μg/mL. As these solutions were not first extracted from blood prior to GC analysis, the estimated concentration of the 'hit' determined from the calibration curve must be adjusted for its recovery from blood. The 'CheckFor' macro refers to recovery data for each drug (Table 7.6) in the *drugs.txt* file and reports an estimated blood concentration (μg/mL).

Table 8.3 reproduces the recovery from blood data which was given in Table 7.6 for each of the drugs detected and quantitated in Reports 8.2–8.5. It also shows the estimated blood level reported by the analyst which was derived from the quantitative data summarised in Table 8.1 for each drug detected. Two estimated blood levels were listed in Table 8.1 for the majority of drugs and the mean value was reported by the analyst in Table 8.3 with an error based on the sum of the standard deviations associated with each extraction procedure. For example, the mean quantitative value for diazepam from liquid-liquid and solid-phase extractions was 0.21 μg/mL (Table 8.1). Table 8.3 shows standard deviations for the two extraction techniques (LLE and SPE) as 4.3% and 3.5% respectively and the error associated with the estimated diazepam blood level is based on their sum (= 7.8%), ie. ± 0.016 μg/mL (Table 8.3; ± 0.02 μg/mL). Paracetamol quantitation was based on calibration standards which did not exceed 5.5 μg/mL, therefore, the analyst reports an estimated figure of "> 5.5 μg/mL (≈ 14 ± 2.5 μg/mL)".

A relatively simple modification to the macro would ensure that the analyst was alerted to blood levels in excess of the highest calibration point being reported by the macro. The 'CheckFor' macro could refer to an additional variable for each drug in the *drugs.txt* file which would list the highest calibration point (eg. 'highpoint'). This could be compared with the concentration determined for the current 'hit' ('CheckFor' macro; quant1) and if it were smaller, the macro could report "quant1 > highpoint".

```
if val(highpoint$) < quant1 then
 if not eqstr(quant$, "") then
   quant$ = quant$ + ","
 endif
 quant$ = quant$ + "> val(highpoint$)"
endif
```

8.4 Discussion

8.4.1 General

Drugs were detected in complicated multi-peak chromatograms on the basis of fixed parameters (RI and a pair of target *m/z* ions) which together uniquely identified each target drug. The macro searched for over 100 drugs in the four blood extracts and estimated the levels of identified drugs using predetermined quantitative information (drug recovery from blood; M and C values from linear regression equations). A report was generated with an *m/z* ion chromatogram for each 'hit', retention time and RI error which allowed the analyst to eliminate false positives and confirm the macro

'hits' as drugs detected. The report provides a hard-copy of drug identification and quantitation in a given blood sample which can:-

1. be produced in court at a later time (eg forensic application),
2. be re-interpreted by an independent analyst,
3. provide a history of the blood analysis, and
4. remove the necessity to archive or store the raw data.

Data acquisition was performed in SIM rather than full scan mode giving greater instrument sensitivity (Section 1.4.2). The drug identification and quantitation macro had significant advantages over the automated techniques described in Section 8.1.

1. It has greater specificity than identification on the basis of drug RI alone as the mass spectrum of the unknown peak must also possess the required pair of characteristic target *m/z* ions. Consequently, an interfering endogenous product or contaminant would be falsely identified as a drug only if it too possessed the two characteristic *m/z* ions. In addition, as four blood extracts were analysed, where appropriate the interfering product must also be identified as the same drug in the corresponding extract (and possibly as a derivative).
2. It is able to identify small peaks which are present at levels close to the limit of instrument detection and 'lost' within a baseline complicated by endogenous blood products (eg. Report 8.5, 7-aminonitrazepam).
3. It is able to identify small peaks on the leading or tailing edge of other larger peaks in the chromatogram.
4. It is able to identify drugs from a mixed mass spectrum produced by co-eluting peaks.
5. It is able to offer confirmation of a drug identification by alerting the analyst to the alternate extract in which the same 'hit' might also be expected.
6. It provides an estimation of the concentration of the drug in blood on the basis of a multi-point calibration curve.
7. It is able to identify drugs in chromatograms generated on any non-polar column (regardless of column length, internal diameter and film thickness, gas flow through the column, oven temperature rate) as macro operation is independent of absolute retention time. The only necessary requirement for successful operation is the chromatography of a series of HC markers.

8.4.2 The Macro

8.4.2.1 *General*

The program consisted of three major macros.

1. 'CustomAnalysis' was the top level macro and directed and initiated other macros ('LocateHydrocarbons,'CheckFor'and 'PickOff'). It opened and closed a path for the generation of the report (#2) and printed part of the final report. It opened and closed a path for the '*drugs.txt*' table (#1) which contained the unique data for each drug used by the 'CheckFor' macro. It established arrays for several variables used in 'LocateHydrocarbons' and 'CheckFor' macros and turned a debugging macro on or off (useful during macro development).
2. 'CheckFor' was the drug detection and quantitation macro and incorporated the minor macros 'TimeToRI' and 'RIToTime'. Using parameters accessed in '*drugs.txt*' this macro chromatographed the two characteristic *m/z* ions for a particular drug over a time window calculated from the drug RI. A drug was

identified if peaks generated from the two *m/z* ion chromatograms coincided to within 0.03 min. The peak area of the one *m/z* ion was used to calculate the drug level in blood (μg/mL). Various analyst warnings and 'fail-safe' mechanisms were incorporated to reduce the number of false 'hits' and errors in quantitation. The 'CheckFor' macro printed part of the final report.

3. 'LocateHydrocarbons' was the macro which identified the HC markers added to each blood extract and which stored the retention time and peak area of each marker for use by 'TimeToRI', 'RIToTime' and 'CheckFor' macros. It created some text and data for the final report. It established the peak area threshold value referred to by the 'CheckFor' macro as $C_{32peakarea}/5000$.

8.4.2.2 *Transfer of the Macro Between Different GC Systems*

Macro operation was independent of absolute retention time and, consequently, could be employed in the automated analysis of chromatograms generated on any non-polar column. From the drug RI values listed in *drugs.txt*, the expected drug retention time could be calculated and was based on linear interpolation between a pair of HC markers which bracketed the drug. The macro searched for target *m/z* ions over a narrow but specific time window. It was shown in Section 3.3 that drug RI did alter slightly in response to altered chromatographic variables. Consequently, although the drug RI values from *drugs.txt* could be employed in the analysis of chromatograms generated on other instruments, an absolute RI error of greater than 25 may be required. Greater macro precision would be obtained by determining the RI of target drugs on individual GC systems (Section 3.4).

The majority of integration parameters incorporated into the macro were independent of GC conditions and instrumentation.

1. The minimum peak area threshold was not an absolute value but set as a fraction of the peak area of C_{32}. It was, therefore, independent of instrument sensitivity. If the operator were to decide that the threshold was set too low, then a single change in the 'LocateHydrocarbons' macro would customise the macro. In the present study, the peak area threshold was set low to ensure drugs present in the blood at low levels were detected. As a result, there was an increase in the number of false positives reported by the macro for the analyst to eliminate (Section 8.4.3.1).
2. The time window over which *m/z* ions were chromatographed was determined from drug RI ± 0.2 min (ie. ± 12 s). If 0.2 min was either too large or small a window on some instruments, a single change to the 'delta' variable in the 'CheckFor' macro would customise the macro.

The parameters set for the integration of 'areaT', however, were very specific and may not be applicable to other GC systems. Integration parameters used to obtain the 'areaT' value were set to accommodate the occurrence of a 'spiky' or tailing peak and ensured its accurate integration. Consequently, if the macro were used to quantitate drugs on other instruments, it is possible that some peaks may be poorly integrated. Similarly, the chromatographic responsiveness of drugs observed on the current GC system may differ on other systems. Therefore, the C and M values listed in *drugs.txt* may no longer accurately describe the linear regression equation which would be fitted to calibration data points on other systems. As indicated for drug RI

values, greater quantitative accuracy would be attained by determining new parameters (C and M values) on individual instruments.

Therefore, automated drug identification (but not quantitation) was independent of specific instrument sensitivity, MS tuning parameters or chromatographic conditions and would be transportable between different GC systems and laboratories.

8.4.2.3 *Macro Operation*

Macro operation was based on correctly identifying the 14 HC markers present in every chromatogram and assigning their retention times. The macro did this on the assumption that the last three peaks in the *m/z* 71.05 profile were C_{32}, C_{34} and C_{36}. If the HC markers were incorrectly identified, the macro would search for pairs of target *m/z* ions at times when the GC method was not acquiring them. The most common outcome of mis-assignment was the "NO DRUGS DETECTED" message.

The only cause of incorrect assignment encountered either during macro development or validation (Chapter 9) was the mis-identification of C_{36} which was the smallest of the HC markers. The macro integrated 20 peaks in the *m/z* 71.05 profile with the greatest peak areas. If more than 20 peaks were chromatographed, it was possible that one of the additional peaks had a greater peak area than C_{36} ('TIC' in Reports 8.2–8.5). Consequently, the macro would overlook C_{36} and mistakenly assign C_{34} as C_{36}. This was observed to occur most often in chromatograms generated from post-mortem blood specimens which contained large putrefaction products (Chapter 9). When mis-assignment occurred, increasing the number of peaks integrated in the *m/z* 71.05 profile from 20 to 21–25 resulted in the correct identification of all the HC markers. The number of peaks routinely integrated in the *m/z* 71.01 profile could be any number (eg. 50) provided that no small peaks between C_{32}, C_{34} and C_{36} were integrated. In general, this was unlikely to occur as the ion trace was very 'clean' in the region of these high boiling compounds ('TIC' in Reports 8.2–8.5).

From the drug RI values listed in *drugs.txt*, the expected drug retention time was calculated and the macro searched for the drug's target *m/z* ions over a narrow but specific time window (drug retention time ± 0.2 min). Within the 'search' window, the macro integrated the peaks with the greatest areas; one peak was integrated for mass1 and the two peaks (if present) were integrated for mass2. Two peaks (if present) rather than one were integrated for mass2, as often the mass2 *m/z* ion was either the less unique and/or less abundant of the target ions. On the basis of peak area alone, the macro may have selected the wrong peak if the multiple peaks were chromatographed over the search window for mass2. As a consequence, the macro would determine that mass1 and mass2 were not coincident (differing by > 0.03 min) and no drug would be identified. For example, the M^+ (mass2) for many of the PFP derivatives of ß-blocking agents and underivatized anti-depressant agents were very small relative to mass1 and chromatographed with relatively small peak areas (eg. alprenolol, Report 8.1). Consequently, if another peak were to elute close by (within the 24 s search window) and also possessed the mass2 *m/z* ion in its full-scan mass spectrum, then the macro may have determined that it had the greater peak area. Similarly, although the HC markers have a negligible contribution of higher masses

in their mass spectra, as the largest peaks in the chromatograms, the macro may still have found that a HC markers had a greater peak area for mass2 than the target drug which was eluting close by. Fewer false negatives were reported, therefore, if the macro integrated two peaks (if present) over the specified time window. Where two peaks were integrated for mass2, the macro identified the correct peak as the one coincident with mass1. If neither peak was coincident, then the macro did not identify a 'hit'.

The macro eliminated some false positives by applying the following criteria. The 'hit' was not reported if the following conditions applied.

1. The RI error was greater than ± 25. In general, an RI error of less than ± 10 was observed for the majority of drugs, but ± 25 RI units was selected as it permitted some variation in the RI of drugs chromatographed in a particular run. It was found during development and validation of the macro that drugs which were chromatographed with poor peak shape sometimes eluted with an absolute RI error of greater than 10 (eg. temazepam, butyl oxazepam). Similarly, the elution of large amounts of drug tended to be retarded by more than 10 RI units (Section 8.1). Drugs which co-eluted with other large amounts of drug were similarly retarded. The elution of large amounts of endogenous material (eg as found in decomposing blood samples) also altered the RI of drugs eluting nearby. It was generally observed, however, that absolute RI did not alter by more than 25 RI units. Therefore, drugs with an RI error greater than 10 but less than 25 were reported as 'hits' which would then allow the analyst to reject or accept them following consideration of the information in the report (Section 8.4.3).
2. The mass1 peak area was less than the peak area threshold. This ensured that false 'peaks' integrated from a 'spiky' base-line were not reported .
3. The peak area of mass2 was less than 0.001 the peak area of mass1. Although some very small mass2 peak areas were to be expected as indicated previously, they were not so small as to satisfy this criterion. This criterion was set to ensure that false 'peaks' integrated from a 'noisy' base-line were not found to be coincident with mass1.

The macro alerted the analyst to the fact that the current 'hit' was coincident with either a HC marker or a previously identified 'hit' (eg Report 8.2; metoclopramide "= C26"; Report 8.3 dihydrocodeine "= #6"). In such cases, the retention times of the two coincident 'hits' were often identical. When the current 'hit' was coincident with a HC marker, it was always the case that the macro had falsely identified the current 'hit'. Drugs susceptible to false identification were those with a $RI_{Drug} = RI_{HCmarker} \pm 25$. As previously stated, although the HC markers had a negligible contribution of higher masses in their mass spectra, as the largest peaks in a chromatogram, the macro sometimes determined that the peak areas of these negligible ions (identical to drug mass1 and mass2) were greater than the area threshold and, of course, coincident. The C_{18}, C_{22} and C_{26} peaks were regularly falsely identified as pheniramine, moclobemide and metoclopramide respectively in underivatised blood extracts (Reports 8.2 and 8.3). When determining the RI for the target drugs and their derivatives (Tables 3.2, 3.4, 4.6 and 5.6), none was found to be exactly the same as the HC markers.

Similarly, when the current 'hit' was coincident with a previous 'hit', it was always the case that the macro had falsely identified one or other of the 'hits' particularly when one 'hit' corresponded to a drug present in the blood specimen. The false identification occurred for exactly the same reasons as a false 'hit' coincident with a HC marker (eg diflunisal was falsely identified in the presence of nortriptyline; Report 8.4). The presence of large amounts of endogenous blood compounds (eg. putrefaction products) sometimes also resulted in the macro falsely identifying drugs (eg codeine and trimethoprim, Report 8.3). The analyst was generally alerted to the presence of endogenous products as large, tailing peaks observed in the full-page 'TIC' with each report (eg Report 8.3). Consequently, the coincident peaks indicated in the macro-generated reports would usually lead to the rejection of some of the identified 'hits'.

During the identification of drugs, the macro searched a narrow time window for the target *m/z* ions. To obtain the peak area used in calculations of drug concentration (areaT), the mass1 peak area was re-integrated under a different set of integration parameters (including peak smoothing) and over a larger time window (chrom AverageTime-(delta/2):AverageTime+(delta*2),mass1). The initial mass1 peak area was not used in the generation of quantitative figures as it often underestimated the peak area due to poor integration. For example, drugs which were chromatographed with a broad or tailing peak shape were sometimes not integrated 'baseline-to-baseline' as the 'tail' lay outside the window over which the *m/z* ion was drawn (chrom rtime-delta:rtime+delta,mass1) and the integrator dropped a perpendicular to the baseline. Similarly, peaks chromatographing at close to the threshold of detection for the instrument were often 'spiky and noisy' rather than smooth peaks (eg. nordiazepam; Table 8.2) such that the integrator often dropped to the baseline at what it perceived was a valley instead of at the tailing edge of the peak. The new integration parameters better integrated the majority of peaks chromatographed including broad, tailing, and 'spiky' peaks.

The drug concentration was determined by the macro from information obtained from *drugs.txt*. The peak area ratio was calculated for each drug (Section 2.6.1) using areaT and the peak area obtained by 'LocateHydrocarbons' for the HC marker specified in *drugs.txt*. The log transformed ratio was then used in the linear regression equation with C and M values specified in *drugs.txt*. These equations were determined from unextracted drug solutions and consequently, an adjustment for drug recovery from blood (specified in *drugs.txt*) was made to calculate the 'quant' variable. The 'quant1' variable ensured that the drug concentration was reported to no more than 3 decimal places. Several analyst alerts were associated with the quantitative figure generated by the macro as follows.

1. The appearance of "¤" indicated that the quantitative figure generated did not relate to the peak area of the 'hit' (see below) and the analyst must perform the integration manually. This occurred most often in relation to false 'hits' with peak areas very close to the threshold area.
2. The appearance of "<" indicated that no recovery data was available for the current 'hit' and 100% recovery was assumed (directly comparable with standard solutions from which the calibration curve was derived). It might be expected that recovery was less than 100% for these drugs and, consequently, the macro would

apply a recovery factor if known (*drugs.txt*). Consequently, the blood level calculated was the minimum possible level.

3. The appearance of "±" indicated that recovery and/or derivatisation of the current 'hit' was very variable from day-to-day and the blood level calculated should be regarded as varying by up to ± 100%.

Occasionally, the macro mistakenly generated a quantitative figure which did not relate to the current 'hit' ("¤"). This can be explained as follows. The macro used a number of variables which were not assigned any value until a 'hit' was identified (eg. time2; time 3). Consequently, the 'CustomAnalysis' macro assigned arbitrary values to these variables (eg. time2 = 200) which were continually overwritten by the value related to the next 'hit' detected. This caused no problems except in rare instances where the macro integrated the wrong peak for mass1 during its re-integration to obtain areaT. On some occasions, the wrong peak was re-integrated because, by extending the time window over which mass1 was drawn (chrom AverageTime-(delta/2) :AverageTime + (delta*2),mass1), an additional peak was found with a peak area greater than that of mass1. The new peak was not coincident with the retention time of the drug (AverageTime) and no new areaT value was determined. However, as the macro still retained the areaT value assigned from the previous 'hit', it went on to calculate a meaningless drug concentration. The analyst was alerted to this problem by '¤'; however, it was also indicated by the mass1 area being identical with that of the previous 'hit' immediately above it in the report. During macro development and validation, this occurrence was observed only for falsely identified 'hits' where there was no real mass1 peak (eg. Report 8.2; dihydrocodeine, thioridazine).

The 'CheckFor' macro printed the part of the report which was related to individual 'hits'. The *m/z* ion chromatogram was drawn, its position on the page was specified and the drug name, retention time, RI error, absolute mass1 area, drug concentration, and other extracts in which to confirm the presence of the drug were given. All the information displayed for each drug was used by the analyst during report interpretation (Section 8.4.3). The absolute mass1 peak area was included should the analyst wish to manually re-calculate the drug concentration or use the peak area in some other way. The macro was designed to display six 'hits' to a page and each page was printed only when the sixth 'hit' was detected. Consequently, the last 'hits' (less than six) were printed by the 'CustomAnalysis' macro.

A 'hit' was identified if the retention times of both mass1 and mass2 were coincident (differing by < 0.03 min). The macro would fail to identify a 'hit' for the following reasons.

1. The peak area of mass 1 was less than the threshold area.
2. More than two peaks were present in the mass1 chromatogram and the 'wrong' peak was integrated as it had the greater peak area; consequently, mass 1 was assigned the wrong retention time.
3. The peak area of mass 2 was less than 0.001 that of mass 1.
4. The mass2 chromatogram had more than two peaks present with greater peak areas than the drug peak; consequently, neither of these had retention times which were coincident with mass1.

5. Integration of either the mass1 or mass2 peaks was poor (eg poor peak shape, weak signal leading to a noisy peak profile) and the resultant retention times were not coincident.
6. The drug RI shifted by greater than ± 25 RI units.

The macro has not been observed to fail as a result of reasons 1, 3 and 4 during either the course of the study or its routine use at the Government Analytical and Forensic Laboratory (GAFL) over 12 months. The macro has been observed to fail, however, in the following two instances.

1. When a very low level of underivatised metoclopramide (RI = 2608, mass1 = 86.10) could not be distinguished by the macro from the large C_{26} peak eluting immediately in front of it (within the 24 s time window); reason 2.
2. When a drug was present at a level close to the detection limit of the instrument and which tended to be poorly chromatographed (eg butyl-oxazepam). In this instance only part of the peak for one mass was integrated due to its spiky peak profile and a retention time was assigned which differed by greater than 0.03 min from the other mass; reason 5. Smoothing the peak caused a greater proportion of the peak to be integrated and assigned a new retention time which was coincident with the other mass. Peak smoothing was not routinely incorporated in the macro (except in the determinatio of areaT), although it would be advantageous for low level drugs.

Consequently, the macro is more likely to fail to detect drugs which chromatograph poorly at levels close to the limit of detection. However, it is unlikely that manual searching of a chromatogram by an analyst would be any more reliable for these low level drugs. For example, the macro clearly identified underivatised nordiazepam in Report 8.2 but visual inspection of the relatively 'clean' mixed *m/z* ion 'TIC' does not indicate a peak (similarly 7-amino nitrazepam; Report 8.5).

The macro has failed on a number of occasions to detect drugs in amounts which caused column overload (reason 6). The therapeutic levels of some acidic drugs are relatively high compared with most basic drugs (Table 6.1). Consequently, during validation experiments (Chapter 9) therapeutic levels of paracetamol, phenytoin and theophylline were often chromatographed with overloaded/distorted peak and their elution was retarded. The 'TIC' from Report 8.4 shows a peak which dominates the chromatogram. This peak was identified as paracetamol by the macro. In some instances, drug RI shifts of greater than 25 RI units were observed and the macro did not report a 'hit'. In such instances, however, the analyst could not fail to observe the large GC peak which was chromatographed for these drugs and which dominated the 'TIC' and suppressed the HC markers. The analyst was immediately alerted to the presence of a significant unidentified peak with an approximate retention time similar to that of either paracetamol, phenytoin or theophylline. The macro could be forced to identify these drugs by temporarily altering the drug RI by approximately 25 RI units in *drugs.txt*. As a result of the overloaded peak shapes, however, any quantitative value generated by the macro was not meaningful. The inclusion of the 'TIC' in the generation of the report is an important safeguard for the analyst against missing a large GC peak.

In the majority of cases, drugs were identified with an absolute RI error of less than 10 RI units. This was consistent with RI errors reported in the literature (Section 8.1) for studies which employed either a homologous series of HC markers (up to 25), or drug or nitrogen-containing markers.

8.4.3 Interpretation of the Final Report

The automated drug identification macro mimicked some of the interpretative skills employed by an experienced analyst and the report generated served as an aid to the analyst. The macro significantly reduced the time required to analyse chromatograms, guarded against the reporting of false negatives when the drug was present at low blood levels, and estimated the approximate level of drugs in blood (μg/mL) which could be used for the future, more accurate, quantitation of that drug if necessary (Section 8.4.4).

The macro, however, did not replace the experienced analyst. As indicated previously (Section 8.4.2.3) the peak area threshold for integration was set very low to ensure that drugs present at low blood levels were identified by the macro. Clearly the low area threshold setting was a contributing factor in the false identification of many of the 'hits' in Reports 8.2–8.5. Consequently, even in a blood sample free from drugs, the macro assigned 'hits' to peaks which resulted, for example, from a noisy baseline. Consequently, the analyst was responsible for correctly interpreting the macro-generated reports and identifying false 'hits' from true.

To interpret a macro report correctly, the analyst applied a 'Check List' of points (Table 8.4) many of which were signalled by Notes and Analyst Alerts listed on each report. A single report generally took less than 1 min of the analyst's time to interpret. An explanation of some of the individual checks listed in Table 8.4 follows and indicates why they are important to the correct interpretation of the macro reports.

1. *Check the correct identification of HC markers.* (Section 8.4.2.2).
2. *Check the 'TIC'*: At a glance the analyst could observe the general chromatography of the HC markers, the cleanliness of the extract, and the presence of large and/or overloaded peaks which potentially have either an altered RI ,or have altered the RI of drugs co-eluting with them.
3. *Check m/z ion chromatograms produced for each 'hit'*: The *m/z* ion chromatograms were of the greatest benefit to the analyst as the validity of 'hits' could rapidly be assessed by eye. With every 'batch' of blood specimens analysed, mixed underivatised and derivatised drug solutions were also chromatographed (Section 2.6.2). Consequently, the analyst had a drug reference with which to compare a 'hit' (refer *ii*, *v*, *vi*). The analyst needed to consider several factors during the examination of ion chromatograms as follow.
   i. 'Hits' must be aligned down the page (Report 8.1) such that off-set peaks were likely to have been falsely identified (large RI error). The pair of target *m/z* ions for a particular drug must have identical peak profiles with each peak beginning and ending at the same time (Report 8.1).
   ii. The peak shapes of the 'hit' and reference drug standard must be similar. Some underivatized drugs are known to be poorly chromatographed (Table 3.2

and 3.4; tailing peak shapes - metoclopramide, nordiazepam, phenindione, primidone, quinine, temazepam). Consequently, if one of these drugs was identified by the macro, it must also have a relatively poor peak shape.

iii. The maximum *m/z* ion abundance (*y* axis) of each chromatogram should be noted. The majority of 'hits' encountered have significantly greater abundances than falsely identified 'hits' (Reports 8.1–8.5) except when the 'hit' is close to the instrument detection limit.

iv. The 'peak' may be an artefact of data acquisition. A change in abundance sometimes occurred as monitoring for one group of *m/z* ions ceased and another began, resulting in a sudden increase or decrease in the baseline. The 'step' produced was sometimes falsely interpreted by the macro as a peak and a 'hit' assigned where RI was appropriate (eg Report 8.2:- benztropine, fenfluramine, thioridazine).

v. The 'hit' may be an interfering endogenous blood and/or derivatisation product (Report 8.4:- indomethacin, theophylline; Report 8.5:- MDMA). In general, interference products were easily identified by the analyst during the interpretation of a 'batch' of blood specimens, as they tended to recur in each extract. Similarly, peak shapes did not match those of reference standards, RI errors were generally large and ion abundance ratios were incorrect.

vi. The 'hit' must have an ion abundance ratio which matches the reference drug standard. Ion abundance ratios were not incorporated into the macro as they are heavily dependent on the tuning of individual instruments. Similarly, the relative abundances of *m/z* ions change with time even on the same instrument. Consequently, the automated search macro was more universally applicable without such a criterion incorporated. In general, 'hits' could be confirmed without specific reference to the relative abundance of the target *m/z* ions. However, a comparison of the 'hit' ratios with the reference standards was useful when eliminating a 'hit'. For example, metoclopramide (Report 8.5) had an incorrect ion abundance ratio.

4. *Check the RI error of the suspected 'hit'.* In the majority of cases, a 'hit' with an error of greater than ±10 could be quickly dismissed (Report 8.4:- indomethacin, theophylline). However, very large to overloaded peaks (concentrations of approximately 1–5 µg/mL) generally eluted later than might otherwise be expected and the late elution was particularly apparent for drugs with lower retention indices (1100 to 1800). For example, regularly encountered social drugs, caffeine, cotinine and nicotine which were present in significant amounts were often identified with an absolute RI error > 10. It was also found that the RI of poorly chromatographed drugs (eg cotinine, nordiazepam, temazepam) were not as reproducible as drugs chromatographed with good peak shape and a retention index shift of greater than ±10 could occur (Section 8.4.2).
5. *Check the suspected 'hit' for coincidence with another peak*: (Section 8.4.2).

If the analyst was satisfied that the suspected 'hit' was real following Checks 1–5, the 'hit' was recorded on the report under "Analyst Interpretation - Drugs Detected". The reports for the remaining three extracts were interpreted in the same manner. The analyst collated the data generated from all four reports (Table 8.2) for further consideration as follows.

6. *Check that a specific 'hit' is present in all extracts indicated by the reports.* As the majority of target drugs would be expected to be found in two or more extracts, this check provides important additional confirmation of the presence of a drug in a blood sample.
7. *Check for the metabolite (if included as a target drug) of a parent drug detected.* The screening procedure included several important drug metabolites (eg. nortriptyline, nordiazepam, THC-COOH) and the presence of both drug and metabolite was strong evidence of the presence of a drug in a blood sample. During validation experiments (Chapter 9) it was observed that in some cases, no drug was present and the metabolite was the only compound detected (eg. THC-COOH, nordiazepam). The identification of only the metabolite might be explained by pharmacokinetic differences between the drug and its metabolite. Consequently, the analyst requires some pharmacological knowledge to best interpret the report findings in relation to drugs and their metabolites such that obvious discrepancies are identified (eg. the occurrence in blood of amitriptyline or diazepam in the absence of nortriptyline or nordiazepam is extremely unlikely).
8. *Check the raw chromatograms if necessary.* In general the analyst would only find it necessary to manually examine the raw data if a report from one extract did not confirm the presence of a drug detected in another and it was suspected that drug RI may have altered as a result of poor chromatography. The possibility that a drug RI had altered as a result of either interference from large amounts of endogenous compounds or an overloaded peak would be indicated in the 'TIC'.
9. *Check the quantitative figure against 'Analyst Alerts'.* The 'CheckFor' macro quantitates drugs detected in each blood extract. Those drugs for which there was no quantitation were indicated by "99.999". Consequently, if a drug was detected in two or more blood extracts, two or more drug blood levels were calculated and reported by the macro. The analyst checks these figures against any 'Analyst Alerts' (Section 8.4.2) and reports the mean value of these figures (Section 8.3.2.2).
10. *Check that quantitative figures in each blood extract are similar.* The recovery data for each drug shown in Table 7.6 give the expected standard deviation from the mean following both solid-phase and liquid-liquid extractions. Consequently, the quantitative values reported for drugs have an error associated with them. In general, less variability in the recovered drug amount resulted from liquid-liquid extraction (Section 7.3.3) and quantitative values from the liquid-liquid blood extract would, therefore, be most accurate. The derivatisation process introduced still further error and, therefore, quantitative figures for PFP derivatives (derived from solid-phase extraction) would incur the greatest error. In general, where the chromatography of the underivatised drug was good, or the reproducibility and/or stability of the drug derivative was poor, the underivatised drug figure was used. Where multiple quantitative figures for a drug were reported each should approximate and confirm the other.

In Section 8.3.3 the falsely identified 'hits' from Reports 8.2–8.5 and reasons for their rejection were listed. Further explanation is required to explain the elimination of trimethoprim and codeine. The apparent trimethoprim peak was a very large overloaded. Trimethoprim is known to chromatograph very poorly, implying that a peak of this size represented drug present at several μg/mL in the blood. However, it

was entirely absent in the liquid-liquid blood extract despite similar, though variable, recovery from blood and this did not confirm the finding in Report 8.3. The analyst interpreted this discrepancy as trimethoprim being falsely identified as a 'hit' in Report 8.3. It was postulated that the very large peak arose from a strongly basic putrefaction blood product which would co-elute with trimethoprim if it were present. This would be extracted under the conditions of solid-phase extraction which extracts strong bases but would be poorly extracted (if at all) by liquid-liquid extraction conditions. Similarly, codeine was detected in the solid-phase blood extract but was unconfirmed in either the liquid-liquid extract or as a PFP derivative in the solid-phase extract. The 'hit' detected in Report 8.3 chromatographed with an exceptionally good peak shape, although codeine generally chromatographs as a slightly tailing broader peak (Table 3.2). Although the recovery of codeine from blood by either solid-phase or liquid-liquid extraction was similar (Table 7.6) and the stability and reproducibility of its PFP derivative good (Table 5.8), the 'hits' identified as codeine in Reports 8.2 and Reports 8.3 and 8.5 were not comparable in either peak area or height. The 'hit' detected in Report 8.3 had both a significant peak area and *m/z* ion abundance of approximately 400000. The 'hits' eliminated by the analyst in Reports 8.3 and 8.5 were much smaller peaks, close to the baseline and with noisy peak profiles indicating that these peaks were close to the limit of instrument detection. Consequently, as with trimethoprim, a strongly basic putrefaction blood product was considered to have been falsely identified as codeine in Report 8.3.

In the routine application of the automated drug identification and quantitation macro, usually, no attempt was made to establish the standard deviation associated with the quantitative figure as described in Section 8.3.3.2. As the relationship between the level of a drug in blood and its effect on an individual is influenced by many factors (Section 10.1), an accurately quantitated blood level may be of no more interpretative value than a semi-quantitative level which indicates that a drug was present at sub-therapeutic, therapeutic, or toxic levels (Section 9.4.1). Therefore, even if the absolute value generated by the macro had an error associated with it of up to ± 100%, in most cases, the blood level could still be placed within the broad therapeutic or toxic ranges described for most drugs (Section 9.4.1). If greater accuracy was required, the blood specimen could be re-analysed and quantitated against a calibration curve which covered an appropriate range as indicated by the macro-generated figure.

8.4.3 Forensic Application and Assessment

The automated drug screening procedure has been in routine use at the Government Analytical and Forensic Laboratory for the past 12 months and applied to over 200 forensic blood specimens. The macro was successfully transported from the development laboratory to the service laboratory with no change made to drug RI in *drugs.txt*. The GC/MS instrumentation was similar to that used in the present study (Section 2.4.1), but there was a direct capillary interface to the MSD giving greater instrument sensitivity. Gas chromatographic parameters were similar to those used in the present study for chromatography with the narrow bore column (Appendix A.2.1). The macro was only used for drug identification.

A full scan acquisition was performed on the underivatised liquid-liquid extract to detect drugs (and pesticides) not included in the target drug screen. Drugs were identified in the TIC chromatogram by automated comparison of individual peaks with both a Hewlett Packard supplied library of drug mass spectra, and an 'in-house' library of drug mass spectra. In general, the TIC chromatogram had many additional small peaks at the baseline compared with the mixed ion chromatogram generated by SIM analyses. To date, the full-scan mass spectral data has not indicated the presence of a drug which had not already been identified by the macro in the underivatised blood extracts. Rarely did the full-scan mass spectral data confirm all drugs identified by the macro in the target SIM analyses. Drugs which were present at relatively high blood levels (eg. cotinine, caffeine, lignocaine, diazepam, methadone) were repeatedly identified in TIC chromatograms. Often, however, no drugs were detected in the TIC chromatograms while the macro indicated that therapeutic levels of drugs were present following target SIM analysis of the same extract. The absence of some drugs in TIC chromatograms which were identified by the macro in target SIM analysis of the same extract indicates the following.

1. Greater sensitivity was achieved through target SIM analyses compared with full scan analyses.
2. The macro was able to identify low levels of drugs amongst general interference at the baseline.
3. The macro was able to differentiate between co-eluting peaks which produced a peak with a mixed mass spectrum which could not be matched against a drug library of full-scan mass spectra.

Currently, three different analysts interpret reports generated by the macro. However, a technician without GC training has difficulty in interpreting the reports correctly. The difficulty arises from a general lack of understanding of fundamental GC concepts and of what the multiple *m/z* ion chromatograms generated from blood extracts represent. Consequently, although the macro has speeded the analysis of complicated chromatograms and reproduced some of the skills that would be used by an experienced analyst, without an understanding of general GC concepts, interpretation of reports is not "fool-proof".

References

1. "Retention indices and dual capillary gas chromatography for rapid identification of sedative hypnotic drugs in emergency toxicology."
Newton B, Foery R; J Anal Tox (1984) 8, 129-134

2. Application of capillary gas chromatography in routine toxicological analyses."
Anderson W, Stafford D; J High Res Chromatogr and Chromatogr Comm (1983) 6, 247-254

3. "Retention indices by wide-bore capillary gas chromatography with nitrogen-phosphorus detection."
Christ D, Noomano P, Rosas M, Rhone D; J Anal Tox (1988) 12, 84-88

4. "A system for toxicological screening by capillary gas chromatography with use of a drug retention index based on nitrogen-containing reference compounds."
Manca D, Ferron L, Weber J; Clin Chem (1989) 35, 601-607

5. "Automated quantitative screening for acidic and neutral drugs in whole blood by dual-column capillary gas chromatography."
Ojanperä I, Rasanen I, Vuori E; J Anal Tox (1991) 15,204-208

6. "Applicability of capillary gas chromatography to systematic toxicological analysis: occurrence of concentration-dependent retention behaviour."
Bogusz M, Wijsbeek J, Franke J, de Zeeuw R: J Anal Tox (1983) 7, 188-192

7. "Automated identification by computer of the mass spectra of drugs in urine or serum extracts."
Smith N; J Anal Tox (1994) 18,16-20

8. "Determination of 1,4- and 1,5- benzodiazepines in urine using computerised gas-chromatography-mass-spectrometric technique."
Maurer H, Pfleger K; J Chromatogr (1981) 222, 409-419

9. "Identification and differentiation of benzodiazepines and their metabolites in urine by computerised gas chromatography mass spectrometry."
Maurer H, Pfleger K; J Chromatogr Biomed Appl (1987) 422, 86-101

10. "Identification and differentiation of β-blockers and their metabolites in urine by computerised gas chromatography-mass spectrometry."
Maurer H, Pfleger K; J Chromatogr (1986) 382, 147-165

11. "Screening procedure for detecting butyrophenone and bisfluorophenyl neuroleptics in urine using a computerised gas chromatography-mass spectrometric technique."
Maurer H, Pfleger K; J Chromatogr Biomed Appl (1983) 272;75-85

12. "Screening procedure for detecting anti-inflammatory analgesics and their metabolites in urine using a computerised gas-chromatography-mass- spectrometric technique."
Maurer H, Pfleger K; Fresenius Z'Anal Chem (1984) 317, 42-52

13. "Automated screening procedure using gas chromatography mass spectrometry for identification of drugs after their extraction from biological samples."
Neill G, Davies N, McLean S; J Chromatogr Biomed Appl (1991) 565, 207-224

Chapter 9.

VALIDATION OF THE AUTOMATED SEMI-QUANTITATIVE BROAD DRUG SCREENING PROCEDURE

9.1 Introduction

An automated semi-quantitative comprehensive drug screen for 105 pharmacologically and structurally diverse drugs in whole blood was developed (Chapters 7 and 8). Target drugs are isolated from the blood matrix through an extraction strategy (combined LLE and SPE) which recovers approximately 90% of target drugs with greater than 75% efficiency. From each blood specimen, four extracts (Figure 2.1) are produced which are analysed by GC/MS (SIM) and drugs or their butyl or PFP derivatives are detected. A macro (Chapter 8) analyses complicated multi-peak chromatograms using fixed parameters (RI and a pair of target *m/z* ions) which together uniquely identifies target drugs. The macro calculates an approximate level in blood for each drug using predetermined quantitative information (Section 8.3.3.2) and generates a report. Validation of this procedure was necessary to evaluate its ability to identify drugs and estimate their concentrations in whole blood.

Three types of blood specimens were selected for validation experiments.

1. Postmortem forensic blood specimens previously analysed using other routine methods by the Government Analytical and Forensic Laboratory (GAFL).
2. Blood specimens distributed to forensic laboratories within Australia and New Zealand as part of a Proficiency Testing Program (PTP) on the identification and analysis of drugs in blood.
3. Blood specimens obtained from hospitalised patients at the Royal Hobart Hospital (RHH) for whom details of drug adminstration (drug, dose, time) were available.

9.2 Methods

Refer to "General Procedure" (Chapter 2).

9.2.1 Blood specimens

All blood specimens were stored at 4°C. Blank blood was obtained from GAFL (Section 7.2). Postmortem forensic blood specimens (66) varied in age (up to two years old) and all were haemolysed. The majority of bloods were in blood tubes with sodium fluoride and potassium oxalate as preservative and anti-coagulant respectively, but some contained no preservative or anti-coagulant. The condition of the blood was also very variable as it was dependent on the nature of the death and the time elapsed after death before blood was taken.

Blood specimens (9) distributed through the PTP on the identification and analysis of drugs in blood also varied in age (up to two years old). The spiked blood sample was prepared by the Division of Analytical Laboratories (DAL) NSW using outdated blood from the NSW Red Cross Blood Bank, spiked with accurately prepared

methanolic solutions of drugs. A blank (drug free) blood sample was also included. The blood samples were distributed to participating laboratories in bottles containing sodium fluoride and potassium oxalate. The spiked and blank bloods distributed through the program to GAFL were tested in validation experiments.

The stability of drugs in the postmortem and PTP blood specimens following an extended period of storage (up to 2 years) at 4°C is not known.

Blood specimens (28) obtained from hospitalised patients at the RHH were selected by a clinical pharmacist on the basis of each patient's current drug therapy and a list of the target drugs. These blood specimens were 7 days old, stored at 4°C and contained whole blood with EDTA as the anti-coagulant. Prior to analysis, the specimens were frozen to haemolyse the blood.

9.2.2 Evaluation of the Automated Broad Drug Screening Procedure.
All blood specimens were analysed blind. Blood specimens were extracted (Figures 2.1–2.5) and analysed (GC/MS; Section 2.4) in an overnight sequence of samples (Section 2.5 and 2.6). In addition to blood extracts (4 extracts for each of 6 blood samples), the sequence included the analysis of:-

1. three mixed reference solutions of drugs (U_{std}) and their butyl (B_{std}) and PFP (P_{std}) derivatives, and
2. the 'performance test mix' (3) which allowed the chromatographic performance of the GC system to be monitored as the sequence progressed (Sections 2.6.2 and 9.4).

The analyst interpreted the macro-generated reports (Sections 2.7 and 8.4.3) which were produced following the automated analysis of chromatograms from each extract and recorded the drugs detected and their estimated concentrations in blood (Table 8.1). The results obtained from the test procedure were compared with those of GAFL and the stated concentrations of the PTP samples. Drug charts relating to the blood specimens from hospital in-patients were obtained by the clinical pharmacist who selected the specimens and drugs detected were compared with the drugs administered.

9.3 Results

9.3.1 Forensic Blood Specimens
9.3.1.1 *Identification*
Seventy-five forensic blood specimens (postmortem and spiked samples) submitted to GAFL for drug analysis were re-analysed using the automated drug screening procedure (Table 9.1). The drugs identified in these specimens by the test procedure and the number of times they were detected in different samples were compared with those identified in postmortem specimens by GAFL techniques and those known to be present in spiked samples. Consequently, data in Table 9.1 indicates the variety of drugs encountered in these specimens and the ability of the screening procedure to repeatedly detect them.

Two broad screening methods had been employed by GAFL to identify drugs in the postmortem blood specimens. The screen for B/N drugs was based on Method 21[1]

(Table 7.2) but with reduced volumes of blood (0.5 mL) and *n*-butyl chloride (1.5 mL) and it was applied to all specimens. The other was a HPLC-DAD method (modified) for A/N drugs[2] but it was not routinely used on all specimens. Some blood samples were forwarded to the Clinical Chemistry Department of the RHH where specific drugs were detected and quantitated through RIA procedures (eg. carbamazepine, salicylates, paracetamol, phenytoin, theophylline, valproate). Similarly, a drugs of abuse screen was sometimes performed on urine specimens at RHH which alerted analysts to the potential presence of amphetamines, cannabinoids, benzodiazepines, cocaine, methadone or opiates in the blood. Selected blood specimens were forwarded to the State Forensic Centre in South Australia for the identification and quantitation of morphine. Therefore, drugs identified by GAFL in postmortem specimens were the results of these combined techniques (when employed) and the ability of the test procedure to identify drugs can be compared with that of the combined GAFL techniques.

Drugs which were known to be present in the PTP samples, and the number of times they occurred in different samples are indicated in Table 9.1. On one occasion GAFL supplied a spiked blood sample for analysis by the test procedure and these are included.

Data in Table 9.1 indicate the following.

1. Fifty-four different drugs and drug metabolites were identified in postmortem specimens (by GAFL and/or the test procedure) or were known to be present in PTP samples.
2. Twenty-seven different drugs and drug metabolites were included in the spiked specimens and some were present in more than one sample. These specimens provided 38 instances for a positive drug identification. Of these, 4 were non-target drugs and, therefore, were not detected by the test procedure (doxylamine, flunitrazepam, midazolam, norpropoxyphene). The test procedure identified all but one of the other target drugs present in the spiked specimens in 33 instances; the exception was metoclopramide (Table 9.1 - Comment 8).
3. No false positives were identified by the test procedure following analysis of the spiked specimens and only one false negative was reported (metoclopramide).
4. Forty-three different drugs and drug derivatives were identified in postmortem specimens by either the test procedure or combined GAFL techniques or both. Of these, 3 were non-target drugs and, therefore, not detected by the test procedure (7-aminoclonazepam, clonazepam, clozapine). Target drugs (excluding caffeine, cotinine, nicotine and theophylline; Table 9.1 -Comment 6), were identified in postmortem samples by the test procedure in 123 instances, and by the combined GAFL procedures in 96 instances.
5. The test procedure identified 9 drugs in postmortem blood specimens which were not identified by the combined GAFL techniques (7–aminoflunitrazepam, 7–aminonitrazepam, doxepin, ibuprofen, naproxen, nicotine, nortriptyline, THC and theophylline).
6. Where the same target drug was identified in postmortem specimens on at least one occasion by both the test procedure and combined GAFL techniques, the test procedure made an additional 22 identifications. The majority of these were in relation to nordiazepam (6), paracetamol (8) and phenytoin (3). Other drugs

identified on single occasions were diazepam, fluoxetine, morphine, oxazepam and oxycodone.

7. Where the same target drug was identified in postmortem specimens on at least one occasion by both the test procedure and combined GAFL techniques, the combined GAFL techniques made an additional 9 identifications. Five of these were in relation to THC-COOH (Table 9.1; Comment 18), one was in relation to sertraline prior to its inclusion as a target drug in the test procedure and, promethazine was once reported as a false positive reported by GAFL (Table 9.1; Comment 14). In two instances, pethidine (Table 9.1; Comment 13) and thioridazine (Table 9.1; Comment 17) were not identified by the test procedure.

Many of the forensic blood specimens were in very poor conditions (green-black in colour; putrid; ranging from fluid to thick or clotted). The LLE technique was able to accommodate any condition of blood without encountering practical problems. The SPE of some of these specimens, however, was troublesome and difficulties were encountered in applying the diluted blood solution to the solid-phase cartridge. For the majority of blood specimens, the diluted blood solution was passed onto the cartridge without the application of a vacuum (Section 2.5.2.4) although the time required to apply the full volume was variable and sometimes slow. For some specimens, the full volume of blood solution could only be pulled through the cartridge under strong vacuum. In one instance, the packing became so occluded that the cartridge was discarded and the procedure begun again with 1.0 mL of blood (instead of 1.5 mL). Following the water wash of the cartridge (Section 2.5.2.5), the passage of all other eluants through any cartridge was comparable despite any previously encountered difficulties.

9.3.1.2 *Estimation of the Concentration in Blood of Identified Drugs*

Data in Table 9.2 compare the drug concentrations estimated by the automated drug screening procedure (Sections 8.3.3.2 and 8.4.3) with those determined by GAFL for the same drugs; on many occassions, GAFL reported a drug as 'present' without quantitation. These data exclude the PTP blood samples where the spiked drug concentration was known (Table 9.3) and drugs identified by the test screening procedure which were either chromatographed with overloaded peak shapes or were present at a concentration greater than the highest calibration point (Section 8.3.3.2).

Drug concentrations reported by GAFL were obtained as follows.

1. External results for morphine (State Forensic Centre, South Australia) and carbamazepine and phenytoin (Clinical Chemistry, RHH) by specific RIA techniques.
2. Semi-quantitative results using either an HPLC technique and a one point (5 μg/mL) calibration curve drawn through the origin, or a GC/MS technique and a one point (1 μg/mL) calibration curve drawn through the origin.
3. Quantitative results using a GC/MS technique and a multi-point calibration curve (but these determinations were rarely performed).

Data in Table 9.2 indicate the following.

1. Similar quantitative results were obtained by the test and GAFL procedures for many individual determinations but there were sometimes inconsistent results

obtained for multiple determinations of the same drug (eg. diazepam and nordiazepam).

2. In all instances where the test and GAFL results differed greatly (Table 9.1; '*italics*'), the GAFL result was always higher than the test result (GAFL > 2x Test).
3. The concentration of diazepam was determined on 13 occasions by the test and GAFL (GC/MS) procedures and was often similar but, in three instances, differed greatly.
4. The concentration of nordiazepam was determined on 9 occasions by the test procedure as the butyl derivative, and by GAFL procedures as the underivatised drug (GC/MS x 8 and HPLC). The results were often similar but, in three instances, differed greatly.
5. Eight specimens were analysed twice (blind) by the test procedure but re-analysis was up to 5 months later (Table 9.2 - Comment 7). The results of duplicate analyses by the test procedure were similar on four occasions (diazepam, methadone, oxazepam, propoxyphene) but paracetamol and phenytoin results differed widely between duplicates. The duplicate results by the test procedure were similar on two occasions to those of GAFL (diazepam, methadone) but the carbamazepine and oxazepam results were consistently lower.
6. The test procedure estimated the concentration of a drug in 60 instances. In 49 instances, the results did not differ greatly (GAFL < Test ± 50%) from those determined by GAFL procedures. In 3 instances where the test and GAFL procedures differed greatly, the result was one of duplicate results for the same specimen but determined 5 months later.

9.3.2 Proficiency Testing Program Blood Specimens

Data in Table 9.3 compare the estimated concentrations of target drugs determined by the automated drug screening procedure (Sections 8.3.3.2 and 8.4.3) with the known concentrations spiked into the PTP blood specimens. These data also include one spiked blood specimen prepared by GAFL which contained drugs at 0.5 μg/mL. Data in Table 9.3 indicate the following.

1. Concentrations in blood were estimated for 19 drugs but, for two drugs, the estimation was not reliable (Table 9.1; '*'); a concentration was estimated in 26 instances.
2. Good agreement (< 10% absolute difference) was obtained between estimated and spiked blood concentrations in 11 instances.
3. A difference of greater than 10% but less than 40% was obtained between estimated and spiked blood concentrations in 12 instances.
4. Poor agreement (greater than 40% difference) was obtained between estimated and spiked blood concentrations for 3 drugs (temazepam, paroxetine, fluoxetine).
5. Approximately 90% of concentrations determined by the test procedure reasonably estimated the true concentration of drug present in the PTP specimens (maximum difference ± 40%).
6. The large difference between the estimated and true concentration of temazepam confirms the finding in Section 7.4.1 which found that the chromatographic response of temazepam was affected by the sample and the current chromatographic performance of the GC system.

9.3.3 Hospital In-Patient Blood Specimens

Data in Table 9.4 list the target drugs administered (drug, route, dose) to 28 hospitalised patients prior to obtaining whole blood specimens for analysis by the test procedure. The drug therapy charts indicated that, of the drugs administered to these patients, 28 were target drugs. Table 9.4 shows the drugs identified by the test procedure in the blood specimens and their estimated concentrations. The time elapsed since the last drug dose is indicated together with the elimination half-life ($t_{½}$) of the drug. The $t_{½}$ value indicates how long a therapeutic level might be expected to persist following the last dose of drug before the blood concentration falls to a sub-therapeutic level. These values, however, provide a guide only as they may be subject to considerable individual variation (dependent on dose, single or maintenance dosing, route of administration, age, sex, liver and kidney function, concurrently administered drugs, etc). For Case No. 17, the time elapsed since the last dose was not specified. The day the blood specimen was taken was known but not the time it was drawn.

Data in Table 9.4 indicate the following.

1. The drug therapy charts listed 28 target drugs which were administered on 112 occasions to hospital in-patients 0 to 5 days prior to their blood samples being drawn.
2. On 64% of occasions where a target drug had been administered, the test procedure identified that drug in the blood specimen.
3. The social drug caffeine was frequently detected in the blood specimens and trace levels of theophylline (a caffeine metabolite) were often detected in the presence of caffeine.
4. In 7 cases, drugs were identified which, according to the corresponding drug charts, had not been recently administered. There were 7 identifications of diazepam and nordiazepam, and 2 identifications of pentobarbitone. Trace levels of diazepam and nordiazepam were consistently detected whenever midazolam (a non-target drug) had been administered. The presence of nordiazepam was not always confirmed in the alternate extract in which it was also expected. In two of these cases (Nos. 2 and 17) pentobarbitone was also identified.
5. In one case (No. 6), 7-aminonitrazepam was identified but, according to the corresponding drug chart, nitrazepam had not been recently administered.
6. On 36% of occasions where a target drug had been administered, the test procedure did not identify that drug in the blood specimen (Table 9.4; 'ND'). One of these occasions can be discounted as the drug was administered orally at the same time the blood specimen was taken (No. 3; morphine).
7. Prednisolone, felodipine and prochlorperazine were orally administered within their approximate $t_{½}$ but were not identified by the test procedure in any blood specimen.
8. The identification of salicylic acid was inconsistent. In only 2 of 5 cases where salicylic acid (100 mg) was orally administered within the approximate $t_{½}$ of the drug, was the drug identified (Table 9.4; Nos. 2 and 9).
9. Following the minimum nightly dose (10 mg), temazepam was consistently not detected approximately 12 h later (the following morning). When a 20–30 mg dose was administered, temazepam was identified 12 h later but the drug was not consistently identified in both underivatised fractions (Table 8.4).

10. Failure to identify felodipine, prednisolone, prochlorperazine, salicylic acid and temazepam at apparent therapeutic blood levels accounted for 33% of reported 'ND'.
11. On 66% of occasions where 'ND' was reported, the time elapsed since administering the last dose was greater than $2t_{½}$ (excluding 2 instances:- morphine; No. 3; nifedipine where time elapsed since the last dose was not known; No. 17) For example, No. 2:- frusemide > $24t_{½}$; No. 4:- metoclopramide > $6t_{½}$,
12. Paracetamol was administered in 23 cases and identified in 19. The test procedure failed to detect the drug (Nos. 16, 20, 21, 24) only when several $t_{½}$ had elapsed since the last dose (18–80 h elapsed) but, in one case (No. 4), it was identified 100 h after the last recorded dose.
13. Drug charts indicated the administration of metoclopramide (10 mg) in 5 cases and it was identified in 2 (Nos. 22, 24). In three instances, the drug was not detected following oral administration where the time elapsed since the last dose was > $2t_{½}$. The drug was detected, however, 18 h later following an intra-muscular administration (No. 24).
14. Drug charts indicated the administration of frusemide (40 and 80 mg) in 8 cases. The drug was not detected in 3 cases and drug charts for two of these indicated that > 60 h had elapsed since the last dose. Frusemide was not detected in Case No. 15 even though the time elapsed since the last recorded dose was within the one $t_{½}$.
15. Estimated drug concentrations appear to reflect therapeutic and sub-therapeutic ranges. For example, large doses of paracetamol (500–1000 mg) are reflected by large blood concentrations (eg 10–20 μg/mL) when the time elapsed since the last dose was similar to $t_{½}$. As the time elapsed since the last dose increased (multiple $t_{½}$), the estimated blood concentration decreased (< 1μg/mL).
16. Estimated drug concentrations at close to the limit of instrument detection may be associated with a large error (Table 9.4; Note '±' and Section 9.4).

It is assumed that hospital administered drugs reflect therapeutic drug use. Consequently, the drug blood concentrations estimated by the test screening procedure should fall within the reported therapeutic ranges for these drugs (Table 6.1). If the elapsed time since the last recorded dose was equivalent to multiple $t_{½}$, then the estimated blood concentration should reflect sub-therapeutic levels. The estimated blood concentrations for the great majority of drugs listed in Table 9.4 were found to be within or below the therapeutic range. Exceptions were noted for 4 drugs in Table 9.4 as follow.

1. Estimated blood levels of salicylic acid were consistently sub-therapeutic.
2. Estimated blood concentrations of morphine (No. 24), nifedipine (No. 8) and metoclopramide (Nos. 22 and 24) were greater than the therapeutic range.

9.4 Discussion

9.4.1 General

In excess of 100 blood specimens were analysed by the automated broad drug screening procedure to evaluate its ability to identify all drugs present in a blood specimen and estimate their levels. During validation experiments, approximately 65

different target drugs and drug metabolites were encountered. The test procedure identified 97% of target drugs known to be present in PTP blood specimens and, in approximately 90% of instances, reasonably estimated their true concentrations (Table 9.3; maximum difference ± 40%). The test procedure compared favourably with methods routinely employed at GAFL for the identification and analysis of drugs in blood and identified a greater number of drugs in the same blood specimens. During the analysis of blood specimens drawn from hospital in-patients, it was found that the test procedure was likely to identify the majority of drugs at therapeutic levels, particularly if administered within $2t_{½}$ of the blood being taken. In addition, by screening for and identifying target drug metabolites, the test procedure provided additional confirmation of the presence of a drug (eg Table 9.1: nortriptyline in the presence of amitriptyline; Table 9.4 No. 26: nordiazepam and temazepam in the presence of diazepam). Drugs and their metabolites were also repeatedly identified at realistic relative concentrations in the blood specimens of drivers involved in road traffic accidents (Tables 10.1, 10.3 and 10.4).

Non-target drugs were encountered in both forensic and hospital blood specimens. To increase the versatility of the test procedure, it is suggested that one of the underivatised extracts be re-analysed in the full-scan mode. If a non-target drug is present in the blood at sufficient levels to permit detection and is able to be chromatographed without derivatisation, it may be identified following GC/MS analysis in the less sensitive full-scan mode (Section 8.4.4). Individual peaks in the TIC chromatogram could be automatically matched against a commercial or in-house library of drug mass spectra (Section 1.2). The re-analysis of an underivatised extract would increase overall analysis times but this could be accommodated in overnight analyses of GC samples. The advantages and disadvantages associated with full-scan and SIM mode analyses are discussed in Section 1.4.2.

It was indicated in Section 7.4 that following analysis of solid-phase extracts, the observed chromatographic performance of the GC system deteriorated less than following the analysis of liquid-liquid extracts. During validation experiments, underivatised extracts from SPE were analysed first followed by underivatised extracts from LLE. The 'performance test mix' (Section 2.2.5.10) was analysed immediately following each set of extracts. In general, the chromatographic performance was satisfactory (Section 2.6.2) following analysis of solid-phase extracts although the absolute peak areas of temazepam, phenobarbitone and high boilers (verapamil and thioridazine) were reduced. Following analysis of the liquid-liquid extracts, often chromatographic performance remained satisfactory (but with a further reduction in the peak areas of some drugs) but, on other occasions, chromatographic performance had deteriorated so much that temazepam and phenobarbitone were absent. This was dependent on the nature of the blood samples analysed (some were severely decomposed). Consequently, it is likely that extracted material from both LLE and SPE techniques contributed to the deterioration of the GC system and frequent and regular maintenance would be required to restore performance.

The drug screening procedure was developed in 2 year old blank blood with sodium fluoride and potassium oxalate as preservative and anti-coagulant, respectively.

During validation experiments, the majority of blood specimens encountered contained these compounds but others contained either no anti-coagulant or preservative, or EDTA as an anti-coagulant (Section 9.2.1). No differences were observed in either drug extraction or macro identification and quantitation when blood specimens which contained either EDTA, or no sodium fluoride and potassium oxalate, were analysed.

9.4.2 Identification of Drugs

Data in Table 9.4 indicated that some drugs could not be detected at therapeutic levels (prednisolone, prochlorperazine, felodipine). Similarly, some drugs present at low levels (eg. temazepam, diltiazem) were detected in only one of the fractions in which these drugs could be expected. It was apparent that the test procedure did not reliably identify all target drugs and confirm their presence when their concentrations in blood were low (Table 9.4; [4]). During the analysis of forensic samples, it sometimes occurred that a peak was present in one extract but was not confirmed in the alternate extract (eg. thioridazine; Table 9.1). It may be possible that these unconfirmed, low level 'hits' were, in fact, unreported drugs. The analyst would have to assess such occurrences on a case by case basis.

In Section 7.4.1, it was indicated that the sensitivity of the procedure to THC-COOH would be decreased as a result of impaired butylation of the drug in the presence of co-extracted blood products. During validation experiments, it was found that the test procedure did not identify THC-COOH in blood specimens when GAFL procedures had determined it was present but at concentrations less than 25 ng/mL. The test procedure does, however, detect THC which is the psychoactive constituent of *Cannabis*. It is the presence or absence of this drug in the blood that has the greater interpretative value as the metabolite (THC-COOH) is not pharmacologically active. Consequently, the limit of detection for THC-COOH by the automated drug screening procedure (approximately 25 ng/mL) is satisfactory and THC may be detected in the apparent absence of its metabolite.

Data in Table 9.1 indicate that morphine was not detected by the test screening procedure in the first forensic specimen which contained it. It was often found that the poor condition of the blood specimens resulted in extracts which were difficult to dry completely without use of excessive heat. It was postulated that the residual water retained in the extract residues hindered the derivatisation of morphine. Consequently, a slight modification to the PFPA derivatisation procedure was introduced using acetonitrile (originally present to solublise the extract residue; Section 5.3.1.1). The residual water was successfully removed through the formation of an azeotrope with acetonitrile (Figure 2.5). Morphine was identified in all subsequent blood specimens which contained the drug and estimations of blood concentration were reliable (Table 9.3).

Data in Table 9.4 indicate that trace levels of diazepam and nordiazepam were repeatedly identified in blood specimens from 7 patients who had been administered midazolam (a non-target drug) and, in two cases, pentobarbitone was also identified. In each case, the midazolam had been administered 3–4 days prior to the blood sample being drawn. The metabolism of midazolam does not produce diazepam or

its metabolite. Similarly, it is not possible that diazepam or its metabolite were formed as artefacts during extraction or GC analysis (particularly since nordiazepam was detected as the butyl derivative). Consequently, it is likely that the patient had been recently administered diazepam and, in two cases, pentobarbitone. The drug therapy charts provided with the blood specimens did not detail the complete drug-taking histories of the patients. In routine procedures at the RHH, drugs administered in the Intensive Care Unit (ICU), Department of Emergency (DEM) and during anaesthesia are not recorded on the drug therapy chart. Further investigation of the medical records belonging to the 7 patients indicated that all had undergone 3 or 4 vessel coronary artery bypass grafting within the last 5–6 days and arrived on the ward from ICU where they had been sedated, intubated and ventilated. 'Omnipon' (morphine derivative) + diazepam are often given (iv) as a crash induction to anaesthesia prior to intubation.[6] Similarly, diazepam is given to premedicate surgical patients.[6] Pentobarbitone is a metabolite of thiopentone - a short acting barbiturate used in anaesthesia.[6] It is likely, therefore, that the midazolam listed on the drug chart was administered to irritable or uncomfortable patients immediately following their surgery. The presence of trace levels of diazepam and nordiazepam indicate the tail end of a dose administered in either ICU or during surgery.

It was indicated in Section 9.3.3 that the estimated blood concentration of four drugs did not correspond well with their therapeutic use in supervised hospitalised patients.

1. Salicylic acid determinations were either consistently sub-therapeutic, or not detected. This may be explained by the very poor recovery of this drug from blood (< 6%; Table 7.6). Consequently, the automated broad drug screening procedure is not suitable for the reliable therapeutic monitoring of salicylic acid or its level in blood.
2. One estimated morphine concentration was well in excess of therapeutic levels and likely to be fatal in non-tolerant individuals. However, it is likely that the estimated drug concentration realistically represented the true concentration in the blood specimen. The specimen was obtained from a patient who had experienced 5 months of pain prior to surgery for the "resection of a paravertebral tumour and reconstruction with a lattismus dorsalis flap and split skin grafting from the thigh" (surgical consultant correspondence included in the patient's medical records). The operation was performed 5 days before the blood specimen was taken and two days before the patient was returned to the ward with a morphine infusion (18 mg/h). The infusion continued for 3 days (decreasing by 2 mg/h each day) and was occurring when the blood sample was taken. The patient was both admitted and discharged with prescriptions for 'MS Contin' (morphine) and morphine elixir. In such a case, it would not be unexpected that the patient had developed considerable pharmacological tolerance to the drug with its long term use and was able to tolerate very large doses of the drug; a dose which might otherwise be fatal to an individual with no previous exposure to morphine. In addition, the automated broad drug screening procedure performed well in estimating the concentration of morphine in the 3 spiked PTP specimens (Table 9.3).
3. Two estimated metoclopramide concentrations exceeded the therapeutic range for this drug. On re-examining drug recovery by the test procedure, data in Table 7.6 indicate that the chromatographic response of metoclopramide was greater when

chromatographed with co-extracted endogenous blood products than when analysed in a reference solution of mixed drugs (Section 7.3.3; P_{std}), resulting in greater than 100% recoveries being recorded. Consequently, the elevated blood levels determined for metoclopramide in Case Nos. 22 and 24 (Table 9.4) are likely to reflect that the drug was chromatographed with a greater response than when the calibration curve was established from reference drug standards (Sections 2.6.1 and 8.3.3.2). In addition, by 'contaminating' the GC system with repeated injection of blood extracts, reference solutions of mixed drugs containing metoclopramide (approximately 1 µg/mL) which were analysed in the same batches as Case Nos. 22 and 24 (Section 9.2.2) were also elevated (> 1 µg/mL). Consequently, estimated metoclopramide blood concentrations calculated by the automated drug screening procedure are likely to be consistently elevated.

4. One estimated nifedipine concentration exceeded the therapeutic range for this drug. It was not unexpected that the estimated concentration would be unreliable as data in Table 7.6 indicate that nifedipine recovery was non-reproducible. Similarly, it was stated in Section 7.4 that, although the screening procedure may be able to report that the drug was present or absent, it could not reliably offer any estimation of the amount present. This was confirmed in the validation experiments.

9.4.3 Estimated Drug Levels

Data in Table 9.2 indicate that, although similar quantitative results were obtained by the test and GAFL procedures for many individual determinations, there were inconsistent results obtained for multiple determinations of the same drug (eg. diazepam). Similarly, duplicate analyses by the test procedure performed up to 5 months apart (Table 9.2) did not always generate similar quantitative results. This inconsistency in quantitative results may reflect the time period elapsed between analyses conducted by GAFL and the test procedure rather than the inability of the procedure to reproducibly estimate blood levels of drugs. It was indicated in Section 9.2.1 that some forensic blood specimens were up to 2 years old. Consequently, the quantitative inconsistencies between GAFL and the test procedure may reflect the deterioration of some drugs in the sample itself after months of storage. This cannot be proven, however, as there was no re-analysis of old specimens by GAFL techniques.

A comparison of quantitative figures generated by GAFL methods and the test screening procedure may not be the best indicator of the ability of the screening procedure to estimate drug concentrations reliably. Unknown errors may have arisen from GAFL methods (eg. use of a one point calibration curve forced through the origin).

Data in relation to the spiked blood samples provide a more valuable assessment of the success of the test screening procedure to identify drugs (Section 9.3.1.1) and estimate their level in blood (Table 9.3). These data indicate that in approximately 90% of instances, the test procedure reasonably estimated the true concentration of drugs in spiked specimens (Table 9.3; maximum difference ± 40%). Of what 'interpretative value' is an estimated drug level in blood with a potential error of up to ± 40% for many drugs and even greater for some (eg. temazepam)?

1. From a clinical perspective, a quantitative result for a drug has meaning only in relation to its clinically effective range in blood; eg. sub-therapeutic, therapeutic, toxic and fatal.
2. From a forensic perspective (Section 1.2) the blood concentration of a drug which is likely to impair judgement or psychomotor skills or both over some critical time period is important only in that it reflects a therapeutic or excessive dose. In fact, the actual concentration of a drug which acts on the CNS is likely to be of less interpretative value than the knowledge that one or more psychoactive drugs are present (Section 10.1).
3. Where significant pharmacological tolerance to a drug can develop with repeated use (Section 10.1; eg. benzodiazepines, methadone, morphine), the estimated (or true) concentration of that drug in blood has little value when compared with the reported therapeutic range as a concentration that would either impair or be fatal to a non-tolerant individual, would have much less effect on a tolerant individual.

Therefore, although quantitative precision is highly desirable, an estimate of the drug level in blood may be no less valuable than an accurately determined figure, particularly when the therapeutic range of a drug is relatively broad. For example, the therapeutic range of propranolol is 0.01–0.34 μg/mL (Table 6.1). Consequently, a blood concentration of 0.07 ± 100% μg/mL still falls within the therapeutic range. However, this is not true of all drugs. For example, data in Table 9.3 show the true and calculated concentrations of fluoxetine as 0.52 and 0.11 μg/mL, respectively. The true concentration represents a potentially toxic dose (but less than reported in fatalities from fluoxetine alone[7]) while the estimated concentration is therapeutic (Table 6.1).[7] Therefore, estimated blood concentrations generated by the test screening procedure with a potential error of up to ± 40% would have to be individually evaluated as to whether or not they could be useful or misleading.

It was indicated in Section 9.3.3 that estimated drug concentrations at close to the limit of instrument detection may be associated with a large error. This error resulted from the following:-

1. the spiky/noisy profiles of the integrated peaks; as the number of samples across the peak was small, a poorly defined peak shape resulted;
2. poor chromatography and adsorption to active sites in the chromatographic system which become more pronounced at levels close to limit of instrument detection (Sections 3.4, 4.4 and 5.4).

In addition, data in Table 7.6 indicated the non-reproducible recovery of some drugs (eg. nifedipine, metoclopramide); therefore, any concentration determined by the test procedure for these drugs should be used cautiously in interpretation.

In Table 9.2, attention was drawn to the quantitative results obtained by the test and GAFL procedures for underivatised drugs, nordiazepam and temazepam. Both these drugs were included in the 'performance test mix' (Section 2.6.2) as they are good indicators of a deteriorating chromatographic system (reflected by tailing peaks with decreased areas). It would be expected that either the HPLC analysis of these drugs, or the GC/MS analysis of the butyl derivative (of nordiazepam) would decrease the likelihood that a variable chromatographic response would occur in response to the changing performance of the GC system. These analyses would more accurately

reflect the true concentration of these drugs in the blood. This expectation was confirmed by data in Table 9.3 which indicated that estimated nordiazepam concentrations (as the butyl derivative) were more accurate than estimated temazepam concentrations (underivatised drug).

More accurate quantitation might be achieved by the test procedure if it were to analyse mixed drug standards (underivatised, butyl and PFP derivatives) at three different spiked concentrations with every batch of blood samples. This would eliminate problems associated with increased or decreased chromatographic response due to matrix effects, response variations associated with the current condition of the GC system and variations in drug recovery. To automate the quantitation, however, a more complicated macro would be required which would use the three spiked standards as 'calibrators', create a calibration curve, calculate C and M values for the linear regression equation and automatically insert them into the *drugs.txt* file for each drug in the mixed standard.

The test screening procedure was perhaps too sensitive an assay for some acidic drugs (eg. phenytoin, paracetamol, theophylline) which are administered in relatively high doses and result in high therapeutic blood concentrations. During analysis of forensic and hospital specimens, overloaded peaks occurred as a result of the large amount of drug injected onto the column and no reliable estimate of drug concentration could be obtained. It could not be determined, therefore, whether or not these drugs were present at therapeutic or potentially toxic levels. In contrast, an overloaded peak identified as a basic drug (Table 9.3; doxepin, propoxyphene) would almost certainly represent a toxic level as a therapeutic drug amount (Table 6.1) is well within the column loading capacity (Tables 3.2, 4.9 and 5.9).

During investigation of drug recovery by the test procedure (Section 7.3.3) it was indicated that the chromatographic response of some drugs was affected by extracted blood products which were co-chromatographed with them. For these drugs, recovery data was more reproducible when the reference standards (U_{std}, B_{std} or P_{std}) were redissolved and analysed in blank blood extracts (Table 7.6). Although the chromatographic response of some drugs (eg temazepam) was adversely affected by their chromatography with extracted endogenous material, the response of others (eg. some drugs in the 'performance test mix') were improved following priming of a clean liner with 1 to 2 injections of an underivatised blood extract (Section 7.3.1); in effect, 'contaminating' the GC system. For example, with repeated injections of blood extracts during overnight analyses of a batch of samples, the response of metoclopramide in a reference solution of mixed drugs was improved (Section 9.4.2). In Sections 3.4, 4.4 and 5.4 it was indicated that some drugs were more susceptible than others to adsorption by 'active sites' in the injector port or on the column and to 'interfacial' adsorption (Section 3.4). This was reflected by decreased peak area and asymmetric peak shape. Extracted drugs represent a very small proportion of the total extracted material present in a sample for GC analysis. Consequently, the improved chromatographic response of some drugs is likely to be due to sites in the GC system which, had adsorbed these drugs when analysed in a reference solution of mixed drugs, and became saturated with extracted blood products when analysed in a blood extract. The expected outcome, therefore, should be improved sensitivity to

these drugs but, during validation experiments, this could not be confirmed as no drugs were quantitated at levels close to their limits of instrument detection (Table 6.1).

During the pilot study (Chapter 10) it was observed that THC was identified at concentrations in blood which were lower than the apparent limit of detection indicated by data in Table 6.1 which were established in chloroform solutions of mixed drug standards. For example, the THC concentration estimated by the screening procedure for Table 10.3-Case No. 7 (Table 10.4) was 10 ng/mL but that determined by GAFL with a deuterated THC internal standard was 7 ng/mL; approximately half the apparent detection limit established for that drug as indicated in Table 6.1. Similarly, Table 10.3-Case No. 6 (Table 10.4) indicated trace levels of THC estimated to be approximately 7 ng/mL. Therefore, THC is one drug for which evidence suggests that a lower limit of detection is possible following its GC analysis in blood extracts compared with analysis in a reference chloroform solution of mixed drugs.

References

1. "Capillary gas chromatographic drugs screen for use in forensic toxicology."
Drummer O, Horomidis S, Kourtis S; J Anal Tox (1994) 18, 134-138

2. "A class-independent drug screen in forensic toxicology using a photodiode array detector."
Drummer O, Kotos A, McIntyre I; J Anal Tox (1994) 17, 225-229

3. "Clarke's Isolation and Identification of Drugs."
A. C. Moffat (Ed) 2nd Edition; The Pharmaceutical Press; London (1986)

4. "Australian Prescription Products Guide."
J Thomas (Ed) 23rd Edition; Australian Pharmaceutical Publishing Company Limited; Australia (1994).

5. "Human Toxicology."
Descotes J (Ed.) 1st Edition (1996) Elsevier Science B.V. Amsterdam; 43-113

6. "Manual of Anaesthesia."
J. C. Snow (Ed) 2nd Edition; Little, Brown and Company; Boston (1982) 11-19.

7. "Disposition of Toxic Drugs and Chemicals in Man."
Baselt R, Cravey R (Eds.) 4th Edition (1995) Chemical Toxicology Institute USA; 337

Chapter 10.

PILOT STUDY – INCIDENCE OF DRUGS AND ALCOHOL IN TASMANIAN DRIVERS INVOLVED IN ROAD ACCIDENTS

10.1 Introduction

10.1.1 General

Over the last 20 years a great deal of literature has been published which discuss the relationship between driving performance and the presence of drugs in the blood of drivers, and the possible causal role of drugs in road traffic accidents (RTA). It is generally accepted that drugs which act on the central nervous system (CNS) have the potential to affect some, or all, of the functions associated with the multi-skilled task of driving. Drug classes with the potential to impair driving can be broadly grouped as follows.[1-3]

1. CNS depressants - sedating types of antidepressants and anti-histamines; benzodiazepines; barbiturates; anti-psychotic agents.
2. Narcotic analgesics - opiate drugs (eg. morphine, methadone, oxycodone).
3. CNS stimulants - amphetamine and its analogues; cocaine, ephedrine isomers; anorectic agents.
4. Cannabis.
5. Hallucinogens - LSD; MDMA.
6. Other therapeutic agents - anti-diabetic, antihypertensive and anticonvulsant agents.

Numerous studies have reported the relative prevalence of drugs in the blood of impaired, injured and killed drivers. In every study, the most prevalent drug/drug group other than alcohol was Cannabis, with CNS stimulants, (especially cocaine), benzodiazepines and opiates varying between second, third and fourth ranking.[1,4-10] The mere presence of centrally acting drugs in the blood of a driver, however, is not sufficient evidence of driver impairment.

Drugs are designed to produce specific pharmacological effects. Despite the specificity of drugs, it is not uncommon to find that in addition to producing the desired pharmacological effect, a drug exerts its effects at other receptor sites and undesirable side-effects become apparent. In general, the desired pharmacological effect is achieved when the drug blood concentration is within the 'therapeutic range'. The therapeutic range is the concentration of drug that provides a therapeutically desirable response in the majority of individuals with minimal toxicity/side effects.[11,12] But it is possible that even healthy individuals may vary considerably in their responsiveness to a drug and underlying diseases/disorders can further alter responsiveness. The same individual may even respond differently to a drug at different times during the course of treatment. This means that should the drug blood level exceed the reported therapeutic range, the individual need not be intoxicated or suffering excessively from drug side-effects. Sources of variability in an individual's response to a drug include individual differences in first pass metabolism, age, gender, weight, degree of obesity, genetic makeup, the

administration of other drugs concurrently, type and degree of disease and prior experience with the drug or structurally-related drugs.[11-13]

For the majority of drugs, reported therapeutic ranges were developed in plasma or serum and not whole blood. Consequently, for a given drug, the level determined in whole blood and the therapeutic range may not be directly comparable. Therefore, because of these sources of uncertainty (inter-individual variation; drug plasma/whole blood relationship), the therapeutic range serves only as a guide to the expected therapeutic response which might be achieved at a particular drug concentration.

An alcohol blood level of > 0.1 g/100 mL in the driver is a significant contributing factor in road accidents.[4,8,14-17] But no similar quantitative data is currently available for any other drug, largely owing to the wide variation observed between drug level and pharmacological effect in different individuals.[3] Consequently, a wide range of drug blood levels may give equal levels of impairment and drug levels producing impairment in one individual may not impair another. For example, one study reported the blood concentration of oxazepam in 14 drivers apprehended by police together with comments from the arresting officer. In each case, no other drug was detected. The blood concentrations covered a wide range from 0.2 - 8 mg/L with gross impairment of the driver indicated by the attending police officer in every case.[5]

Some drugs have different pharmacological effects at different blood concentrations. For example, diazepam has a muscle relaxant effect at one concentration and is an anti-convulsant agent at higher blood levels.[18] The pharmacological effect of lignocaine is dependent on the route of administration; subcutaneous injection produces local anaesthetic effects while the intravenous injection of lignocaine produces an anti-arrhythmic response.[18]

With repeated use of the same drug, tolerance can develop to the side-effects and sometimes the therapeutic effects of the drug, and so larger and/or more frequent dosing may be required to produce the same pharmacological effects. Consequently, therapeutic blood levels in tolerant individuals could greatly exceed reported therapeutic ranges. Not only may tolerance develop to the drug being used but so may cross-tolerance to structurally similar drugs. Therefore, a tolerant individual with a drug blood level which might be considered toxic or even lethal to an intolerant individual may exhibit no signs of impairment and proper control of a motor vehicle may be expected.[19] For example, morphine, a narcotic analgesic and CNS depressant, has the capacity to impair driving performance. In intolerant individuals, morphine causes sedation, affects mood and impairs reaction time, muscle coordination, attention and short-term memory.[20] One study compared driving ability in cancer patients receiving long term morphine analgesia with a control group of cancer patients. Cancer patients receiving long term morphine analgesia can achieve very high blood concentrations of morphine exceeding cited therapeutic ranges. Yet it was found that morphine had only a very slight and selective effect on some functions related to driving in the group receiving morphine, compared with its effect on the control group, because of the remarkable

pharmacological tolerance these patients had developed to the side effects of morphine.[20] It is noted, however, that the group of cancer patients receiving long term morphine analgesia regularly received the same dose of morphine. Should an individual omit doses or increase the dose, then this irregularity in the dosing regimen may prevent the development of pharmacological tolerance and so the possibility of drug impairment remains.[19]

In summary, then, the presence of drug/s in the blood of an individual is not sufficient evidence of impairment because of the possibility of individual differences in response to the same blood drug concentration. In addition, individuals with diseases such as epilepsy, schizophrenia, anxiety etc. may present a greater risk to other road users if they were to go untreated than if they were taking their antiepileptic, antipsychotic or antidepressant medications.[2,21-25]

In 1983 a consensus panel representing the disciplines of clinical pharmacology, analytical and forensic toxicology, law and forensic medicine concluded that:-

> The major value of [drug blood level] determinations is to suggest that demonstrated clinical impairment of function is probably related to the presence of the drug in the body fluid, in the absence of other reasonable explanations. Conversely, impairment of function without the presence, in significant amounts, of a drug that might be expected to produce impairment would strongly suggest some cause, unrelated to drug use.[21]

Consequently, a drug blood level alone cannot establish driver impairment but in conjunction with other evidence (eg. eye witness accounts, medical examination and an estimation of the impairing potential of the drug/drug combination) it can help support or exclude a diagnosis of driving impairment due to a specific drug.

Despite the potential for centrally acting drugs to impair driving performance, in most cases where these drugs are taken as directed, significant or prolonged impairment is unlikely. The period where impairment is likely to be greatest is during the initial stage of taking a prescribed drug; before the nature and extent of any intended or side-effect is known with respect to the individual concerned, and before tolerance develops.[19,27] Therefore, impairment is usually only likely where a single drug is taken in excess or two or more CNS affecting drugs are taken together.[1]

Blood alcohol concentration is relatively easily assessed at the road side with breath determinations of alcohol. Poor driving performance or unusual or inappropriate behaviour, however, constitute the only signs of possible drug intoxication for the arresting officer. One study[1] reported that in 90% of cases where police officers specially trained in recognising signs of drug impairment said they suspected impairment, one or more psychotrophic drugs was present.[1] Another study[10] reported that traffic police who were not trained in drug recognition often concluded that accidents involving drivers who were subsequently found to be drug but not alcohol positive, were caused by lack of driver care and attention. Ideally, the assessment of both the officer and the examining doctor would be required to identify drug impairment but, if the driver was unconscious as a result of serious injury following the accident, then eye-witness accounts of possible driver impairment prior

to the accident would be necessary. In addition, it may be virtually impossible for the police to distinguish between driver impairment by drugs and by injury (shock, concussion etc). Consequently, injury in drug impaired drivers tends to protect them against successful prosecution.[5,27] It has been reported that police were reluctant to request blood and urine samples unless very strong visual evidence of impaired driving performance was available.[5]

The causal role of drugs in road accidents has been difficult to establish as there are driver-related factors other than potential impairment of the driver by drugs which are likely to contribute to the occurrence of road accidents. For example, driver age, sex, personality, confidence, psychomotor skills, driving experience, risk perception and acceptance all interact to affect individual driving performance.[2,16,22] Road accidents may arise as a result of other contributory factors which are unrelated to driving performance such as the type, size and road worthiness of the vehicle; road conditions; time of day; weather; other traffic; location (urban/rural; curve/intersection).[2,10,16,22] For example, one comprehensive study investigated potential drug impairment in fatally injured truck drivers.[8] The study included detailed on-site accident investigation and found that, in the absence of drugs, multiple factors (eg. driver fatigue, inexperience, medical problems, failure to heed warning signs or to yield, mechanical problems and load shifts) contributed to the fatal accident. It is not surprising then, that several studies have found that large percentages of drivers involved in road accidents (injured/fatally injured) were drug and alcohol free as follows:- 42%[4], 43%[10], 43%[17], 45%[7] ,51%[28], 66%.[8] These percentages further indicate, however, that many drivers were not drug and alcohol free at the time of the accident.

The possibility that influences outside driver control contributed to an accident has been recognised[4,14] and, in many recent studies, a driver responsibility analysis has been conducted in which each driver is rated for responsibility in causing the accident. If more drugs were detected in drivers assessed as responsible for accidents than in drivers not responsible, then this would be considered evidence of driver impairment by drugs. It is now generally accepted that the risk of having a road accident increases with increasing blood alcohol[1,7,14,17,27] and subsequent studies based on driver responsibility analysis confirm this.[4,8,14,28] Alcohol consumption is implicated as a major factor in fatal motor vehicle crashes, and there is a clear association between the seriousness of the crash and alcohol involvement.[4] Although driver responsibility analysis determined that driver impairment by alcohol was causal in road accidents, the same analysis has still not consistently indicated impairment caused by other drug groups (Cannabis, tranquillisers, anti-histamines, opiates, stimulants).[1,4,8,14,28,30] For example, results from separate studies employing responsibility analysis do not agree that Cannabis use impairs the driver although no results were statistically significant because of the small number of Cannabis positive drivers detected.[1,4,8,28,31]

The low incidence of individual drugs other than alcohol in drivers makes trends suggesting driver impairment, as indicated by responsibility analysis, difficult to validate statistically. Such studies have indicated, however, that drivers with multiple drugs (excluding alcohol) in their blood who are involved in accidents are

more likely to be responsible for the accident than drug free drivers or drivers with a single drug (excluding alcohol) in their blood.[1-4] The responsibility rates for these drivers was similar to those of alcohol positive drivers (alcohol either alone or in combination with another drug).[1] In addition, these types of studies indicate that drug positive drivers are more likely than drug free individuals to be involved in an accident, in particular, a single vehicle accident.[4,8,22,29]

There are few, if any, consistent findings in the current literature relating any specific drug or drug group to driver impairment. Apparent from the literature is that alcohol, alcohol + drug, or multiple drugs, either impair or have the potential to impair driving performance, and increase driver responsibility rates for accidents.[1-4]

10.1.2 Drugs and Tasmanian Drivers

The most recent analytical study of the incidence of drugs in Tasmanian drivers involved in RTA was performed in 1987.[32] The study involved the analysis of 200 whole blood samples taken from road users for alcohol analysis over a 20 month period by the Tasmanian Police. The blood samples represented all drivers killed in road accidents during that period and drivers that had come to the attention of the police who would not accept the alcohol breath test reading or who were incapable of being breath tested. A further 387 blood samples were taken from donors at two Tasmanian Blood Banks as the control group.

Drug analysis was performed by gas chromatography on a slightly polar capillary column and drugs detected were confirmed by GC/MS. The analytical procedure was designed to detect lipophilic drugs which would be expected to have effects on the CNS. Basic drugs were extracted at pH 9.6 into *n*-butyl chloride (Table 7.2; Method 1) and chromatographed without derivatisation. Solid phase extraction at pH 1.5 extracted acidic drugs which were methylated prior to chromatographic analysis. Cannabinoids in blood were screened by RIA and confirmed by GC/MS.

Ninety-six reference drugs (not listed) were chromatographed which included sedative-hypnotic, antidepressant, antipsychotic, anticonvulsant, analgesic, anti-histamine and nonsteroidal anti-inflammatory agents. The study, however, did not determine whether all these drugs were able to be extracted from blood by the two extraction procedures employed or, if they could be detected at therapeutic levels. The study reported that the screening procedure did not detect two important illicit drug groups (opiates and amphetamines) or some commonly occurring licit medications (paracetamol, salicylates and some antidepressants). It is also postulated that many other commonly prescribed medications containing weakly acidic and basic drug would also be excluded from the screen due to their poor extraction at pH 1.5 or 9.6.

Alcohol > 0.05 g/100 mL was detected in 75% of blood samples taken from road users involved in road accidents. The study detected 34 drugs other than alcohol in 194 drivers, bicycle riders and pedestrians, with cannabis being the most frequently identified.[32] In the control group, 16% contained alcohol but none exceeded the legal limit of 0.05 g/100 mL. From the control group, only one blood sample contained a drug (Cannabis) other than caffeine. The study recognised that, although

blood donors were not an appropriate control group for a driving study, had their pattern of drug use been similar to that of road users involved in road accidents, then it could have been concluded that drugs were not contributing to accidents. The findings of the study, however, did not support a similar pattern of drug use between the control and test groups.

10.1.3 Analytical Techniques

The broad drug screening procedure will be used in a pilot study to investigate the incidence of drugs in Tasmanian drivers involved in road accidents (1995 to 1996) but, more importantly, to assess whether the procedure is readily applicable to these types of studies. As indicated previously (Section 1.4.1) broad drug screening is commonly achieved through a combination of techniques which target specific drug groups and employ a variety of instrumentation. Many of the studies cited in Section 10.1.1. adopted this approach to the identification and confirmation of drugs in blood. For example, one major study[4] screened for drugs in blood using SPE with GC/MS analysis and RIA and confirmed their presence with either HPLC, GC-ECD or immunoassay. A minimum of 6 mL of blood was required for the complete drug assay which encompassed approximately 50 drugs. The screen included alcohol and LSD which are not identified by the procedure developed in this study. Other studies employed techniques which identified specific drug groups in blood using, for example, enzyme-multiplying techniques, HPLC, GC and GC/MS.[6-8,10] Still other studies were based on drugs identified in the urine of drivers following an accident.[19,27,29] Clearly, if the screening procedure developed in this study was applicable to the study of drugs in drivers, it could significantly minimise the resources (sample volume, staff, instrumentation, time) required by laboratories undertaking such studies and may increase the number and diversity of drugs they can currently detect.

10.2 Methods

Refer to "General Procedure" (Chapter 2).

Blood specimens submitted to GAFL for analysis were re-analysed by the automated broad drug screening procedure. The specimens were taken from fatally injured drivers (33) or obtained under the Road Safety (Alcohol and Drugs) Act from drivers involved in road traffic accidents (16). The majority of bloods had been stored (up to 16 months) at 4°C with sodium fluoride and potassium oxalate as preservative and anti-coagulant respectively. Some blood specimens from fatally injured drivers, however, contained either no preservative or anti-coagulant, or EDTA as an anti-coagulant (Section 9.4).

10.3 Results

Table 10.1 lists the drugs and/or alcohol detected in the blood of fatally injured drivers involved in road traffic accidents (RTA) over a 16 month period (September 1995–December 1996). Not all blood specimens belonging to fatally injured drivers over this period were re-analysed by the automated drug screening procedure. The specimens excluded were those for which there was an insufficient volume of blood

remaining for re-analysis, or those from drivers who underwent an extended period of hospitalisation prior to death. Consequently, data in Table 10.1 includes 7 of the 12 driver fatalities in the 1995 period and 26 of 37 fatalities in the 1996 period.

From additional written information available about each accident (included in the coronial reports, and police reports to the pathologist and to the Accident Investigation Unit of the Department of Transport), an assessment was made as to whether or not the driver was responsible for causing the accident. Table 10.2 gives examples of the statements and circumstances included in the reports which enabled the drivers to be assigned as 'Responsible', 'Partly Responsible' or 'Not Responsible' in Table 10.1. In general, drivers assessed as responsible for the accident failed to maintain their designated lane on the road. For those drivers who were found to be partly responsible, poor weather or road conditions, driver inexperience, inattentiveness or fatigue, or an unroadworthy vehicle also contributed to the accident.

Associations between variables were assessed by chi-squared tests (X^2). It should be noted, however, that for the resulting theoretical distribution to be reasonably accurate, "the expected number of individuals in all cells must be at least 5."[33] The data in Table 10.1 does not satisfy this assumption as the number of individuals in the three categories are small with the 'Not Responsible' group containing only 5. Also, some drugs were excluded from analysis as not being likely to impair driving (eg. paracetamol). Consequently, the probabilities (P) resulting from the X^2 statistics suggest trends rather than significant differences between variables.
Data in Table 10.1 indicate the following.

1. Regardless of drug findings, an alcohol concentration greater than the legal limit of 0.05 g/100 mL was identified more frequently in blood from drivers who were responsible for the accident than for those who were either partly, or not, responsible as shown below; $X^2 = 9.144$; df = 2; P = 0.0103.

Findings	Responsible	Partly Responsible	Not Responsible
Alcohol > 0.05[1]	13	6	0
Alcohol < 0.05[1]	6	3	5

[1] 0.05 g/ 100 mL of blood.

2. All drugs detected in the blood specimens of driver, except paracetamol, were able to affect the CNS[1,18,21,36,37] or indicated the use of a drug which affects the CNS (eg THC-COOH).

3. Although the screening procedure is capable of identifying a wide range of drugs in blood, the drugs identified in the blood specimens of 'responsible' drivers were limited to drugs of abuse, benzodiazepines, anti-depressants, anti-convulsants, non-steroidal anti-inflammatory agents (and paracetamol).

4. Of the 33 fatally injured drivers, approximately one third were female. No difference was observed in the relative proportion of females within each responsibility category (X^2 =0.405; df = 2; P = 0.817).

5. Drugs (which affect the CNS) and/or alcohol (> 0.05 g/100 mL) were identified more frequently in blood from drivers who were responsible for an accident than for those who were either partly, or not, responsible; ie. A to C versus D below; X^2 =16.536; df = 2; P = 0.0003.

	Findings	Responsible	Partly Responsible	Not Responsible
A	Alcohol[1] alone	8	1	0
B	Alcohol[1] + Drugs	3	0	0
C	Drugs alone	5	1	0
D	No alcohol[1]/drugs	3	7	5

[1] Alcohol concentration > 0.05 g/100 mL of blood.

6. Single vehicle (SVA) rather then dual vehicle (DVA) accidents tended to occur more frequently in the responsible and partly responsible driver categories than in not responsible driver category as shown below; X^2 =8.005; df = 2; P = 0.0183).

Findings	Responsible	Partly Responsible	Not Responsible
SVA	13	6	0
DVA	6	3	5

7. No difference was observed between the 3 responsibility categories in the age of drivers as the upper and lower 95% confidence intervals (CI) overlapped each other as shown below.

Age (years)	Responsible	Partly Responsible	Not Responsible
mean (SD)	34 (15)	26 (15)	43 (22)
n	19	9	5
CI (95%) upper	41	37	71
lower	26	15	15

Table 10.3 lists the drugs identified in the blood of drivers involved in RTA which the attending police officer/s suspected were impaired by drugs. These data, therefore, represent a select group of drivers and exclude the following.

1. Those involved in accidents which the police did not attend.
2. Those which had a high enough breath alcohol reading to explain any signs of impairment which were recognised by the police.
3. Those in which signs of impairment were not recognised by the police.
4. Those which were unconscious immediately after the accident (in these cases, police usually only request that the blood be analysed for alcohol).

Full details were not available or were not able to be obtained for all accidents recorded in Table 10.3 ('-'). In cases where an elapsed time was recorded, the minimum elapsed time was 1¼ h. It was assumed, therefore, that the elapsed time for Case Nos. 7, 9 and 14 was at least 1¼ h.

Data in Table 10.3 indicate the following:

1. In every case where the attending police officer/s suspected drug impairment, CNS affecting drugs or their metabolites were identified.
2. Although the screening procedure is capable of identifying a wide range of drugs in blood, the drugs identified in the blood specimens of these drivers were drugs of abuse, benzodiazepines, anti-depressant agents, ibuprofen and paracetamol.

3. More than one CNS affecting drug was identified in the blood of 7 of 16 drivers and, in two cases, alcohol was also present.
4. Cannabis use was indicated by the presence of THC or its metabolite in the blood of 11 drivers. Despite obvious impairment at the time of the accident which was recognised by the attending police officer/s, in approximately half of these cases, the level of THC in the blood was below the level of instrument detection 1¼–5½ h after the accident.
5. Benzodiazepines were detected in the blood of 7 drivers.
6. Anti-depressant agents were identified in the blood of 3 drivers.
7. Three of the 16 drivers were female.
8. A greater number of the accidents were SVA although the accident type was not specified for all cases.

Table 10.4 lists the estimated concentrations of drugs which were identified and quantitated by the automated drug screening procedure in Tables 10.1 and 10.3. These data confirm the finding in Chapter 9 which indicates that the developed procedure could reliably estimate sub-therapeutic, therapeutic and toxic levels of drugs in blood as demonstrated by the following examples.

1. Eye-witness accounts of driving impairment (Table 10.3) would suggest either ingestion of drugs at greater than therapeutic levels where undesirable side-effects are manifested, or ingestion of multiple drugs where the possibility exists for potentiation of the pharmacological effects of the individual drugs. Where police had suspected that drugs were impairing an individual's driving ability, data in Table 10.4 indicate that licit drugs were present at therapeutic and toxic levels 1¼ to 2 h after the accident.
2. Where benzodiazepines (diazepam and temazepam) and their metabolites were detected, the screening procedure estimated relative blood levels which were similar to those reported in the literature[33,34] following the metabolism of ingested diazepam and temazepam.
3. The blood specimen analysed in Table 10.1–Case No. 2 was taken 42 h after the accident during the individual's hospitalisation. The estimated drug levels determined by the screening procedure accurately reflect the expected levels for drugs present; those administered in hospital (diazepam and morphine) were present at therapeutic levels and those taken prior to the accident (phenytoin and carbamazepine) were present at sub-therapeutic levels.

10.4 Discussion

A pilot study investigating the incidence of drugs in the blood of Tasmanian drivers involved in RTA was undertaken to:-

1. demonstrate one application of the broad drug screening procedure, and
2. revisit the types and frequency of drugs identified in the blood of Tasmanian drivers as determined in 1983 by an earlier study.[32]

Although the sample size was very small, the sample revealed findings that are consistent with data reported by other studies as follows, and this consistency lends some confidence to the reliability of the data from the pilot study.

1. Drugs (which affect the CNS) and/or alcohol (> 0.05 mg/100 mL) were identified more frequently in blood from drivers who were responsible for the accident than from those who were partly, or not, responsible.[4,8,22,29]
2. Single vehicle accidents rather then dual vehicle tended to occur more frequently in the responsible, or partly responsible, driver categories than in the not responsible, categories.[4,8,10,22,29] .
3. In every case where the attending police officer/s suspected drug impairment, CNS affecting drugs or their metabolites were identified.[1] The drugs detected in the blood of apparently impaired drivers and their pattern of use were similar to those found in other studies; Cannabis use was prevalent; it was common to either administer multiple centrally acting drugs concurrently or administer a large amount of a single drug.[1]

The similarity between results determined in the pilot study and those reported in the literature validates the useful and successful application of the screening procedure to a study which investigates the incidence of unknown drugs in a limited volume of blood. A small number of centrally acting drugs was detected in driver blood despite the ability of the screening procedure to identify a diverse range of drugs. Although the data did not contribute anything new towards clarifying the 'drugs and driver' issue so far described in the literature, these unsurprising results lend credibility to the data determined by the developed screening procedure.

Data in Table 10.1 were grouped in relation to assessed driver responsibility in causing the accident. This study found that alcohol (a known risk factor for road accidents) was associated with driver responsibility. Consequently, this finding supports the assignment of driver responsibility as assessed from information included in the coronial reports, and police reports to the pathologist and to the Accident Investigation Unit of the Department of Transport.

The data determined in this study was similar to that determined by an earlier study[32] suggesting that the types of drugs (drugs of abuse, sedative-hypnotic agents, drugs taken for the relief of pain and inflammation) identified in the blood of Tasmanian drivers has changed little in 10 years. Anti-convulsants, antidepressants and paracetamol which were detected in the present study were, however, not reported in the previous study and this difference may be as a result of the different analytical methods employed by each study. The earlier study reported that the drug extraction procedure which was employed did not recover paracetamol and some antidepressants and it was postulated in Section 10.1.2 that other drugs (eg. weakly acidic drugs such as the anticonvulsants) might also be poorly recovered.

As a result of the extremely limited sample size of this study, the frequency with which drugs were detected in the blood of drivers cannot be compared with the findings of the earlier study. A comprehensive study into the incidence of drugs in the blood of Tasmanian drivers involved in RTA which utilises the developed screening procedure has the potential to expand on the previous study[32] undertaken in Tasmania as follows.

1. It would permit identification of a broader range of target drugs including those for which the previous study did not screen (eg. amphetamines, opiates, anti-depressants, anti-convulsants, paracetamol).
2. It would be able to detect the majority of target drugs within their therapeutic ranges.
3. It would be able to reliably estimate drug concentrations which may reveal their therapeutic use or abuse.

References

1. "The effects of drugs (other than alcohol) on road safety: 1st report incorporating collected papers."
Road Safety Committee, Victoria Aust. (1995) L.V. North Government Printer,1-29

2. "Drugs and driving sensible recommendation for your patients."
McLean S; Current Therapeutics (1987) 35-46

3. "Special issue drugs and driving."
Accident Anal Prev (1985) 17, 281-353

4. "The incidence and role of drugs in fatally injured drivers - Final report."
Terhune K, Ippolito D, Hendricks J; National Highway Traffic Safety Administration US Department of Transportation (1992) Report No. DOT HS 808 065147

5. "Experiences in relation to drug/driving offences."
McLinden V; J Forensic Science Society (1987) 27, 73-80
Australian.

6. "Driving under the influence of other drugs than alcohol in Norway."
Gjerde H, Christophersen A, Bjorneboe A; Forensic Sci Int (1990) 45,5-14

7. "Alchohol and drug use among motor vehicle collision victims admitted to a regional trauma unit: demographic, injury and crash characteristics."
Stoduto G, Vingilis E, Kapur B; Accid. Anal. and Prev.(1993) 25,411-20

8. "The prevalence of drugs and alcohol in fatally injured truck drivers."
Crouch D, Birky M, Gust S; J Forenisc Sci (1993) 38, 1342-1353

9. "Detection of cannabinoids in homicide victims and motor vehicle fatalites."
Garriott J, Vincent J, Di Maio M; J Forensic Sci JFSCA (1986) 31, 1274-1282

10. "Alcohol, drugs and impairment in fatal accidents in Brotish Columbia."
Mercer G, Jeffery W; Accid. Anal and Prev. (1995) 27, 335-343

11. "Clinical Pharmacokinetics. Concepts and Applications."
Rowland M, Tozer T (Eds.); (1980) Lea and Febiger, Philadelphia, 160

12. "Basic and Clinical Pharmacology."
Katzung B (Ed); (1987) 3rd Edition; Lange Medical Publications. USA, 18

13. "Individual variation in first-pass metabolism."
Tam Y; Clinical Pharmacokinetic Concepts (1993) 25, 300-328

14. "Responsibilty analysis: a methodology to study the effects of drugs in driving."
Robertson M, Drummer O; Accid. Anal and Prev. (1994) 26, 1 - 5

15. "The role of the drinking driver in traffic accidents (the Grand Rapid study)."
Borkenstein F, Crowther R, Shumate R; Blutalkohol (1974) 11 (Suppl1)

16. "Factors affecting the drinking driver."
Chan A; Drug and Alcohol Dependence (1987) 19, 99-119

17. "Incidence of drug and alcohol intake in road traffic accident victims."
Vine J, Watson T; med J Aust. (1983) 1, 612-15

18 "Goodman and Gilman's the Pharmacological Basis of Therapeutics."
Hardman J, Limbird L (Eds. in Chief) 9th Edition (1996) The McGraw-Hill Companies USA, 1712 - 1792

19. "Experience with drugs and driving in Queensland, Australia."
Wilson D;Med Sci Law (1985) 25,2-10

20. "Driving stability in cancer patients receiving long term morphine analgesia."
Vainlo A, Ollila J, Rosenberg P; Lancet (1995) 346, 667-70

21. "Drug concentrations and driving impairment."
Consensus Development Panel; J American Med. Assoc. (1985) 254, 2618 -21

22. "Mad drivers: Psychiatric illness and driving performance."
Cremona A (1986) British J Hosp Med (1986) 193-195

23. "Cognitive-psychomotor functions with regard to fitness for driving of psychiatric patients treated with neuroleptics and antidepressants."
Gerhard U, Hobi V; Neuropsychobiology (1984) 12, 39-47

24. "The effect of antipsychotic drugs on driving and driving related psychomotor functions."
Judd L; Accid Anal and Prev (1985) 17,319-322

25. "Drugs and driving."
O'Hanlon J, de Gier J (Eds);1986 1st ed. Taylor and Francis Inc. USA, 17-68

26. "Prescription drugs and driving performance."
Lancet (1984)112

27. "The impaired driver: Hospital and police detection of alcohol and other drugs of abuse in motor vehicle crashes."
Orsay E, Doan-Wiggins L, Lewis R; Annals of Emergency Medicine (1994) 1,51-55

28. "Drugs in drivers killed in Australian road traffic accidents."
Drummer O; Victorian Institute of Forensic Pathology Monash University (1994) report No. 0594

29. "Alcohol and drug use among motor vehicle collision victims admitted to a regional tauma unit: Demographic, injury and crash characteristics."
Studuto G, Vingilis E, Kapur B; Accid Anal and Prev, (1993) 25,411-420

30. "Are benzodiazepines a risk factor for road accidents?"
Benzodiazepine/Driving Collaborative Group; Drug and Alcohol dDependence (1993) 33,19-22

31. "Marihuana and driving."
Moskowitz H; Accid Anal and Prev. (1985) 17,323-345

32. "Drugs, alcohol and road accidents in Tasmania."
McLean S, Parsons R, Chesterman R et. al.; Med. J Aust. (1987) 147, 6 - 1

33. "Primer of Bio-Statistics. International Edition."
Glantz S; Kaufamn B, White J (Eds.) 2nd Edition (1989) McGraw-Hill Book Co. Singapore; 125.

34. "Disposition of Toxic Drugs and Chemicals in Man."
Baselt R, Cravey R (Eds.) 4th Edition (1995) Chemical Toxicolgical Institute, USA

35. "Clarke's Isolation And Identification Of Drugs."
Moffat A.C (Ed.) Second Edition (1986) The Pharmaceutical Press, London.

36. "1996 MIMS Annual"
Carroll, P (Chairman of Editorial Board) 20th Edition -Australian (1996) Tien Wah Press (Pte.) Ltd. Singapore

37. "Australian Prescription Products Guide."
Thomas J (Ed) 23rd Edition (1995) Australian Pharmaceutical Publishing Company Limited; Australia.

Chapter 11.

GENERAL CONCLUSIONS

An automated semi-quantitative comprehensive drug screen for 105 pharmacologically and structurally diverse drugs in whole blood was developed. Target drugs were isolated from the blood matrix through an extraction strategy (combined liquid-liquid and solid-phase extraction) which recovered approximately 90% of target drugs with greater than 75% efficiency. From each blood specimen, four extracts were produced which were analysed by GC/MS (SIM) and drugs, or their butyl or PFP derivatives, were detected. A novel macro analysed complicated multi-peak chromatograms using fixed parameters (retention index and a pair of target *m/z* ions) which together uniquely identified target drugs. The macro calculated an approximate level in blood for each drug using predetermined quantitative information and generated a report.

In excess of 100 blood specimens were analysed by the screening procedure to evaluate its ability to identify all drugs present and estimate their levels. During validation experiments, approximately 65 different target drugs and drug metabolites were encountered. The test procedure identified 97% of target drugs known to be present in spiked blood specimens and, in approximately 90% of instances, reasonably estimated their true concentrations. The screening procedure compared favourably with methods routinely employed at Government Analytical and Forensic Laboratory for the identification and analysis of drugs in blood and identified a greater number of drugs in the same blood specimens. During the analysis of blood specimens drawn from hospital in-patients, it was found that the procedure was likely to identify the majority of drugs present at therapeutic levels, particularly if administered within two half-lives of the blood being taken. In addition, by screening for, and identifying, target drug metabolites the test procedure provided additional confirmation of the presence of a drug.

Chromatographic information was obtained for a wide range of drugs and their derivatives and enabled a database to be established which recorded the observed behaviour of these analytes on a non-polar chromatographic system (retention index, peak shape, linearity over a defined concentration range, limit of instrument detection). From these data it was possible to estimate and compare the apparent 'chromatographic polarity' of individual drugs. Comparisons were made between the chromatographic properties of underivatised and derivatised drugs and their structural characteristics which permitted the formation of either butyl or PFP derivatives. In particular, it was found that some unusual derivatisation products were formed following reaction with PFPA. These were generally not formed at 1° or 2° amine groups on a drug but at alcohol or 1° amide groups. These data add to the published data which deals mainly with either selected drugs, or single drug groups, and is not able to be compared between groups. The drugs studied were representative of a wide range of chemical structures and functionalities. Consequently, the chromatographic properties of other drugs and their suitability to

participate in derivatisation reactions with either *n*-butyl iodide or PFPA may be estimated provided they are structurally similar to a drug already investigated.

A pilot study investigating the incidence of drugs in the blood of Tasmanian drivers involved in RTA was undertaken to demonstrate one application of the broad drug screening procedure. Although the screening procedure was capable of identifying a diverse range of drugs, a limited number of centrally acting drugs was detected in driver blood (drugs of abuse, benzodiazepines, anti-depressants, anti-convulsants, non-steroidal anti-inflammatory agents and paracetamol). Findings determined in the pilot study were similar to those previously reported in the literature. The consistency of data between the pilot and other studies suggests that the screening procedure can be reliably employed in studies which investigate the incidence of unknown drugs in a limited volume of blood. If laboratories undertaking such studies were to employ this procedure, it is possible that the resources they require could be significantly minimised while increasing the number and diversity of drugs they can currently detect.

In the design of any drug screening procedure, the particular needs and limitations of the laboratory in which it was to be employed would need to be accommodated (Section 1.2). In this study, the needs and limitations of a Tasmanian forensic laboratory were considered and it was indicated that a drug screening procedure which satisfied the nine criteria identified in Sections 1.3 and 1.4.1 would be highly desirable. It can be concluded that the screening procedure developed in this study did successfully satisfy, or satisfy in part, the 9 criteria as follows.

1. The drug screen was developed in a limited volume (2.5 mL) of whole, haemolysed blood.
2. The procedure extracted a large number of available drugs with at least one representative drug from each of the major drug classes (eg. benzodiazepines, anti-depressants, anti-epileptics etc.) but, of the drugs 'flagged' in Table 1.2 for inclusion in a broad drug screening procedure designed for both clinical and forensic applications, 120 drugs were eliminated. Approximately 40% of those eliminated were drugs which were commonly used in the community (eg. mineral supplements, hormonal medications, antibacterial and antifungal agents, and non-systemic medications) but which did not satisfy any of the other selection criteria (potential drug of abuse; toxicologically significant; possessing pharmacological properties with the potential to effect driving performance or judgement; a marker of an underlying disorder). The elimination of these drugs, therefore, is likely to impact more significantly on the potential clinical application of the drug screening procedure rather than on its forensic application.
3. The procedure was able to detect the majority of target drugs at therapeutic blood concentrations.
4. The study included data which indicated which drugs could or could not be recovered by the extraction technique and listed the extraction efficiency of the procedure for each drug.
5. Drug recovery was relatively high (90% of target drugs recovered with greater than 75% efficiency). The recovery of only 6% of tested drugs by either SPE or LLE (or both) was less than 30%, and all were acidic drugs. Baclofen and captopril were not recovered by SPE or LLE techniques. The variability in the

recovery of many test drugs was greater following solid-phase than liquid-liquid extraction.

6. The procedure was able to provide an indication of whether the drug was present in the blood at sub-therapeutic, therapeutic or toxic levels in both forensic specimens and blood from hospitalised patients.
7. The drug screening procedure was able to confirm the identity of 60% of the target drugs by an alternate method; either in an extract produced by a different technique (SPE or LLE), or as the corresponding butyl or PFP derivative. The identity of nearly all drugs which would be detected in an underivatised extract, and approximately half the drugs detected as either butyl, or PFP derivatives, could be confirmed in another extract.
8. The majority of target drugs were chromatographed with good peak shape and with a limit of instrument detection which fell within or below the reported therapeutic range of the drug.
9. The macro developed for automation of data analysis provided greater specificity than commercially available library-based search programs and mimicked some of the interpretative skills which would be employed by an experienced analyst The macro was able to identify low levels of drugs amongst general interference at the baseline and was able to differentiate between co-eluting peaks which produced a peak with a mixed mass spectrum (which could not be matched against a drug library of full-scan mass spectra).

The novel macro developed in this study was independent of absolute retention time and, consequently, could be employed in the automated analysis of chromatograms generated on any non-polar column. From the drug RI values listed in *drugs.txt*, the expected drug retention time could be calculated and was based on linear interpolation between a pair of HC markers which bracketed the drug. The majority of integration parameters incorporated into the macro were independent of GC conditions and instrumentation. Therefore, automated drug identification (but not quantitation) was independent of specific instrument sensitivity, MS tuning parameters or chromatographic conditions and was able to be transported between different GC systems and laboratories (transferred from the development laboratory to the service laboratory). The automated drug identification macro incorporated some of the expertise of the experienced operator and the report generated by the macro served as an aid to the analyst. The macro significantly reduced the time required to analyse chromatograms, guarded against the reporting of false negatives when the drug was present at low blood levels, and estimated the approximate concentration of drugs in blood which could be used for the future, more accurate, quantitation of that drug if necessary. The macro, however, did not replace the experienced analyst who was responsible for correctly interpreting the macro-generated reports and identifying false 'hits' from true.

The screening procedure developed in this study appears well suited to forensic work and has been in routine use at the Government Analytical and Forensic Laboratory for the past 12 months. During validation experiments it was found that the procedure correctly identified drugs present in spiked blood samples distributed through the Proficiency Testing Program indicating that it is capable of performing as well, or better than, other forensic laboratories in Australia and New Zealand

which undertake similar analyses but which use a combination of different techniques and instrumentations. At the Government Analytical and Forensic Laboratory, the procedure has been routinely used to analyse specimens submitted by the Tasmania Police and Coroner where it has been necessary to determine the presence of unspecified drugs in a limited volume of blood. Confirmation of the identity of approximately twenty-five drugs detected as butyl and/or PFP derivatives has been recently achieved by combining, silylating and re-analysing the 'S' and 'L' extracts for trimethylsilyl (TMS) drug derivatives. Through silylation, the identity of some high priority target drugs can be confirmed (eg. benzoylecgonine, morphine, oxazepam, temazepam, THC and THC-COOH). The additional step was easily incorporated into the automated screening procedure once the retention index and *m/z* ions of TMS derivatives had been determined and a new *drugs.txt* table created. Total analysis time was increased by a further 25 min. It is possible that some target drugs eliminated in Section 6.3 may be detected as TMS derivatives at therapeutic blood levels, but this has not yet been investigated.

The screening procedure was applied to a limited number of clinical specimens (blood from hospitalised patients) during validation experiments. Many of these were blood specimens from surgical patients and, as a result, only a limited number of target drugs (28) were encountered. To evaluate whether the procedure is suitable for routine clinical applications, blood specimens from patients on different hospital wards (eg. medical, psychiatric) would need to be analysed.